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14. ABSTRACT:

This project is a Partnering PI option with Dr. Jianguo Cheng at CCF as Initiating PI and Dr. Tingyu Qu at UIC as Partnering PI. The specific aims are to generate functional chromaffin-like cells (CLCs) from mesenchymal stem cells (MSCs) and to investigate the analgesic and anti-tolerance effects and the safety of CLCs in animal models. We have conducted the proposed experiments as outlined in SOW. Specifically, we have produced CLCs by reprogramming human MSCs (hMSCs) with the extracts of porcine adrenal chromaffin cells. We have harvested bone marrow tissues from rats and isolated, cultured, and expanded rat MSCs (rMSCs) for the targeted reprogramming by using cellular extracts of porcine adrenal chromaffin cells to produce CLCs. Spinal injection of human CLCs (hCLCs) in rats produced significant antinociceptive effects. No tumor formation and abnormal cell growth were found in SCID mice with hCLC transplantation, suggesting the safety of these cells and their parent cells, i.e., hMSCs, as therapeutics. Recently, we found that even naïve MSCs at their early passages (<passage 5) had significant analgesic and robust anti-tolerance effects in both cellular and animal models. Our research has led to 5 poster presentations at conferences, 3 publications, one published patent, and 2 manuscripts currently in preparation.

15. SUBJECT TERMS:
Mesenchymal stem cells, Cell reprogramming, Pain management, Tolerance, Drug abuse, Cell cultures, Spinal transplantation of autologous stem cells, Animal behavioral tests

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1. Introduction:

Pain is a leading cause of disability among active duty and retired military personnel. Ineffective treatment often leads to pain-related impairments and drug abuse with long-term costs to both the military health and disability systems. Clinical trials have demonstrated that transplantation of allogeneic adrenal chromaffin cells provides significant pain relief in patients with intractable cancer pain and in patients experiencing allodynia, a hallmark of neuropathic pain (1, 2, 3, 4). Following adrenal chromaffin cell transplantation, there is often a long-term pain relief without analgesic tolerance (one year in humans) (5), application of exogenous opiates can further alleviate pain without dosage escalation (4); i.e., there was a stabilization of exogenous analgesic intake in these patients, strongly indicating that transplantation of adrenal chromaffin cells ameliorated the problem of opioid tolerance (2, 6). These adrenal chromaffin cells release a "cocktail" of endogenous analgesic substances, including enkephalins, catecholamines, gamma aminobutyric acid, indolalkylamines, and other neuropeptides (7, 8). The analgesic effects of chromaffin cells can be partially reversed by intrathecal injection of either the opioid antagonist naloxone or the adrenergic antagonist phentolamine (9, 10, 11), suggesting that these effects are mediated largely by opioids and catecholamines released by these cells. The anti-tolerance analgesic effects produced by the transplantation of adrenal chromaffin cells may be attributed to a synergistic action of endogenous analgesic molecules released by the transplanted chromaffin cells.

However, clinical practice has been hindered due to the limited availability of suitable human adrenal tissue, genetically well-matched donors in particular, to serve as grafts. Mature chromaffin cells are post-mitotic when they produce enkephalines and catecholamines. Thus, the expansion in culture of these cells is not possible. Xenogeneic materials, such as bovine and porcine chromaffin cells, have been extensively studied as potential alternative materials to human chromaffin cells. Transplantation of these xenogeneic cells into the spinal subarachnoid space produces antinociceptive effects on both Aδ and C fiber-mediated responses in a thermal pain model of rat and non-human primate (12, 13, 14, 15), with a gradual decline in analgesic efficacy that can be prolonged by administration of immunosuppression (13, 15), suggesting that xenogeneic chromaffin cells elicit host immunological rejection to the transplants, and that immunosuppressive therapy is necessary for enhancing long-term graft survival to extend the analgesic effect of the transplants. In addition, there has been concern regarding pathogen contamination of these xenogeneic materials, such as bovine spongiform encephalopathy for bovine chromaffin cells. Thus, the ideal cell source would be the autologous chromaffin cells derived from the patient's own tissue.

Emerging cell reprogramming technology allows production of chromaffin-like cells (CLCs) from autologous stem cells (16, 17, 18, 19), which can be generated epigenetically to secrete analgesic substances, anti-inflammatory factors, and immunological modulating molecules, and used for the management of chronic pain and prevention of drug abuse. In our preliminary studies, we have successfully generated functional chromaffin-like cells by reprogramming human mesenchymal stem cells (hMSCs) with the cellular extracts of porcine chromaffin cells (20, 21, 22). We hypothesized that transplantation of these reprogrammed CLCs, autologous CLCs in particular, will produce significant long term analgesic and anti-tolerance effects without any major adverse effects and immunological rejection. This project is a Partnering PI option with Dr. Jianguo Cheng at CCF as Initiating PI and Dr. Tingyu Qu at UIC as Partnering PI. They work jointly on the Specific Aims to generate CLCs from hMSCs and rat MSCs (rMSCs) and to investigate the analgesic and anti-tolerance effects and the safety of CLCs in comparison with naïve MSCs in cell cultures and animal pain models. The success of this project is anticipated to establish an innovative therapy that will have a profound impact on pain management and drug abuse prevention, two of the major barriers facing the military and society. Such a therapy will fundamentally reduce the need for exogenous opioid medications and minimize the risk of prescription drug abuse and addiction. It can be used in hundreds of thousands of patients with a wide range of cancer and non-cancer pain states to improve quality of life and save billions of dollars for the military health and disability systems.

2. Key Words:

Mesenchymal stem cells, Autologous stem cells, Cell reprogramming, Pain management, Tolerance, Cellular model, cAMP, Met-enkephalins, Drug abuse, Cell cultures, Spinal transplantation, Animal behavioral tests

3. Overall Project Summary

The specific aims are to generate functional chromaffin-like cells from mesenchymal stem cells (MSCs) and to investigate the analgesic and anti-tolerance effects and the safety of newly-generated chromaffin-like cells in animal models. We have conducted the proposed experiments as outlined in SOW. Specifically, we have produced chromaffin-like cells (CLCs) by reprogramming human MSCs (hMSCs) with the extracts of porcine adrenal chromaffin cells and found that spinal transplantation of these chromaffin-like cells of human origin produced significant analgesic effects in rats. We have harvested bone marrow tissues from rats, isolated, cultured, and expanded rat MSCs (rMSCs) for the targeted reprogramming by using cellular extracts of porcine adrenal chromaffin cells to produce chromaffin-like cells of rat origin, as we performed previously on hMSCs. Recently, we found that naïve hMSCs at their early passages (passages 3-5) demonstrated potent anti-tolerance effects in cellular model, with more Met-enkephalin release than those hMSCs at their late passage (≥passages 10). More importantly, naïve MSCs showed significant analgesic and robust anti-tolerance effects in animal pain models. Safety evaluation of human CLCs generated reliable data in severe combined immunodeficiency (SCID) mice with human CLC transplantation, showing that transplanted hCLCs do not lead to tumor formation or abnormal cell growth in SCID mice during the evaluation period of up to 6 months. The Overall Project Summary specific for the partnering PI's tasks conducted in Qu lab is described below.

1: Generate functional chromaffin-like cells: i.e. to use porcine chromaffin cell nuclear and cytoplasmic extracts to reprogram hMSCs and rMSCs to produce analgesic CLCs. We have produced human CLCs (hCLCs) by reprogramming hMSCs with the extracts of porcine adrenal chromaffin cells (24) based on our previously established protocol (20, 21, 22). reprogrammed hMSCs and non-reprogrammed rMSCs at early stage (<passage 5) and late stage (>passage 10) were isolated, cultured, expanded, and these cells were shipped to Dr. Cheng's lab according to experimental needs for in vivo cell transplantation studies.

<u>1a. Acquisition of porcine chromaffin cells for CLC production:</u>

Porcine adrenal glands (x62) were purchased from Sierra For Medical Science (Whittier, CA), and all of the porcine adrenal glands were processed for chromaffin cell isolation. The protocol used to isolate porcine adrenal chromaffin cells from these adrenal glands is the same as we employed previously (20, 21, 22, 24). In brief, adrenal glands were manually perfused three times with warmed (37°C) Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 5 mM HEPES (Sigma, St. Louis, MO), pH 7.4, supplemented with 100 UI/ml penicillin, 100 mg/ml streptomycin, and 0.125 ml/ml fungizone (PSF; Sigma, St. Louis, MO), followed by digestion of connective tissue with 0.125% collagenase A (Boehringer Mannheim, Mannheim, Germany) in Locke's buffer for 3 × 5 min at 37°C. At the end of the digestion period, the chromaffin cells were isolated from the dissected medulla by mechanical dissociation. The harvested cells were filtered through a 70-μm nylon mesh (BD Falcon, Bedford, MA, USA) and then centrifuged at 150 × *g* for 10 min in Locke's buffer. The resulting cells were purified on 39.47% Percoll gradients (Pharmacia Biotech, Uppsala, Sweden) by centrifugation at 22,500 × *g* for 20 min. The portion of the gradients containing purified chromaffin cells was harvested by aspiration and washed three times by centrifugation at 150 × *g* in Locke's buffer. Freshly isolated

chromaffin cells were suspended and plated in 75 cm^2 culture flasks (Corning, Cambridge, MA) containing Dulbecco's modified eagle medium/F12 (DMEM/F12, 1:1; Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma, St Louis, MO) and antibiotics (PSF). These chromaffin cells were maintained at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA) fed by replacing culture media twice per week and used for experiments within four weeks after culturing because mature chromaffin cells are post-mitotic and do not survive longer in cultured condition.

1b. Cell reprogramming:

hMSC culture: hMSCs were purchased from Cambrex (Walkersville, MD) and AllCells (Emeryville, CA). These cellsare negative for surface markers associated with hematopoietic cells [e.g., cluster of differentiation 11b (CD11b), CD33, CD34, and CD133 antigens] and used for the designed experiments. hMSCs were cultured and expanded using the protocol previously developed in our laboratory (20, 21, 22, 24). In brief, hMSCs were plated in 75 cm^2 culture flasks (Corning, Cambridge, MA) at a concentration of $1x10^5$ cells/cm^2 and cultured in 20 ml growth medium consisting of DMEM (Gibco, Grand Island, NY), an antibiotic-antimycotic mixture (1:100, Invitrogen, Carlsbad, CA), FBS (Stem Cell Technologies, Vancouver, BC, Canada), and incubated at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA). Cells were passaged by incubating with 0.05% trypsin–EDTA (Gibco) for 5 min at room temperature to gently release the cells from the surface of the culture flask after reaching about 80% confluency. Culture medium was added to stop trypsinization, cells were centrifuged at 350 × *g* for 5 min at room temperature, re-suspended, and transferred into new culture flasks at a concentration of 1×10^5 cells/ cm2 for continuous culture and expansion to reach a sufficient number of cells for experimental uses. hMSCs that underwent less than 10 passages were used for the experiments.

rMSC culture: Totally, sixteen adult rat bone marrow tissues (Sprague-Dawley, SD, male) were obtained from Dr. Cheng's lab, and all of the rat bone marrow tissues were successfully processed for rMSC isolation, culture, and expansion. In brief, mononuclear bone marrow cells were isolated and incubated at 37°C in a 5% CO_2 humidified incubator. Homogeneous rMSCs were received based on their adherence to plastic in culture and expanded in culture. The conditions for culturing and expanding rMSCs as well as cell passaging were similar to those used for hMSCs. Sub-populations of cultured rMSCs at early stage (<passage 5) and late stage (>passage 10) were collected, respectively, and shipped to Dr. Cheng (Initiating PI) for cell transplantation experiments.

Reprogramming MSCs **(including hMSCs and rMSCs)** with the extracts of porcine chromaffin cells: The cell reprogramming processes for producing hCLCs and rCLCs are similar.

a. Preparation of porcine chromaffin cell extracts: Cultured porcine chromaffin cells were counted and washed in PBS and in cell lysis buffer (20mM HEPES, pH 8.2, 50 mMNaCl, 5mM $MgCl_2$, 1mM dithiothreitol, and protease inhibitors) (Sigma, St Louis, MO), sedimented at 400×*g*, resuspended in 1 volume of cell lysis buffer, and incubated for 30 min on ice. Cell samples were then sonicated in 200µl aliquot on ice with a pulse sonicator (PowerGen 125, Fisher Scientific) in short pulses until all cells and nuclei were lysed, and confirmed by microscopic observation. The lysate was centrifuged at 15,000×*g* for 15 min at 4°C. The supernatant was aliquoted and stored in liquid nitrogen for later use.

b. Permeabilization of MSCs with SLO: MSCs were suspended, washed in Ca^{2+}- and Mg^{2+}-free PBS, and centrifuged at 120×*g* for 5 min at 4°C. The collected MSCs were resuspended in aliquots of $1x10^5$ MSCs/100µl of Ca^{2+}- and Mg^{2+}-free PBS in 1.5 ml tubes. Cell samples were permeabilized with streptolysin O (SLO; Sigma-Aldrich, St Louis, MO) at a final concentration of 200ng/ml and incubated in

an H_2O bath at 37°C for 50 min with occasional agitation. The cell samples were then placed on ice, diluted with 200µl cold PBS, and sedimented at 150×*g* for 5 min at 4°C.

c. Reprogramming MSCs with the extracts of porcine chromaffin cells: The permeabilized MSCs ($1x10^5$) were resuspended in 100µl of the extracts of porcine chromaffin cells in a 1.5ml tube containing an ATP regenerating system (1mM ATP, 100µM GTP, and 1mM of each NTP, 10mM creatine phosphate, 25µg/ml creatine kinase) (Sigma, St Louis, MO). Cell samples were incubated in an H_2O bath at 37°C for 1 hr with occasional agitation. To reseal the membranes of MSCs, 1ml of DMEM containing 2mM $CaCl_2$ and antibiotics were added to the tube and incubated at 37°C for an additional 1 hr. Finally, $CaCl_2$-containing DMEM was replaced by fresh DMEM with 10% FBS and reprogrammed MSCs were transferred to culture flasks at a concentration of $1x10^5$ cells/cm^2 and expanded continuously for subsequent experiments.

1c. Characterization of CLCs:

Task 1c for hCLC production is completed and the data have been published (24):

Gene expression of hCLCs: One week after cell reprogramming with the cellular extracts of porcine chromaffin cells, RT-PCR was performed to examine the expression of hPPE. The molecular size of the RT-PCR product for the human preproenkephalin (hPPE) gene, a precursor for enkephalin opioid peptides was 425bp. As expected, naïve hMSCs demonstrated a low level of inherent hPPE gene expression. The chromaffin-like cells generated from the reprogrammed hMSCs showed a significantly enhanced expression profile for gene hPPE compared to that of naïve hMSCs ($P<0.01$), suggesting that cell reprogramming further increases the expression of hPPE genes in the population of reprogrammed hMSCs.

Immunocytochemical examination of hCLCs: Following cell reprogramming, morphological changes in hMSCs were observed within the first few days; i.e., reprogrammed hMSCs became smaller and rounder. Five days later, the cells reverted to fibroblast-like shapes. The expansion of these cells in culture was at a slightly slower speed (doubling time: about 84 hours) in the first week and recovered at a normal dividing rate (doubling time: about 72 hours) similar to that of naïve hMSCs. Two weeks after cell reprogramming, immunocytochemical examination showed that most of the resultant chromaffin-like cells (≥90%) expressed a strong immunoreactivity for Met-enkephalin and TH, specific cytoplasmic markers for adrenal chromaffin cells. Interestingly, BrdU-positive staining was detected in a subpopulation of chromaffin-like cells, suggesting that some chromaffin-like cells may have retained a similar proliferative capability as that of hMSCs and could be expandable in cell cultures.

However, the generation of rCLCs from rMSCs by using the same methods as employed for the generation of hCLCs from hMSCs is not so successful. The main reasons may include that 1) the cultured rMSCs we used for producing rCLCs grew much slower in culture than hMSCs, 2) it took much longer to obtain sufficient amount of rMSCs for producing rCLCs, and 3) the reprogrammed rMSCs easily became senescent in culture.

1d. The phenotypic stability and secretory function hCLCs:

Phenotypic stability of hCLCs: These hCLCs remained stable phenotypes and expanded in cultures with an average cell doubling time of about 72 hours up to one month, undergoing about 8 passages. We did not maintain the culture of the cells post the one month time point after cell reprogramming.

Secretory function hCLCs:

a. Immunoblot: In parallel to the time point examined for the expression of hPPE genes by RT-PCR (one week post-cell reprogramming), the serum-supplemented culture medium for hCLC and naïve hMSC cultures was replaced by a serum-free culture medium. Twenty-four hours later, the medium was collected and purified for Met-enkephalin detection by immunoblot assays. The level of Met-enkephalin released by hCLCs was significantly augmented compared to that released by naïve hMSCs for the same number of cells ($1x10^5$ cells/well) in serum-free cultures ($P<0.01$). Although naïve hMSCs were able to produce and release a low level of Met-enkephalin into the serum-free medium, augmented production and secretion of Met-enkephalin opioid peptides in hCLCs were consistently observed in each of the four independent experiments.

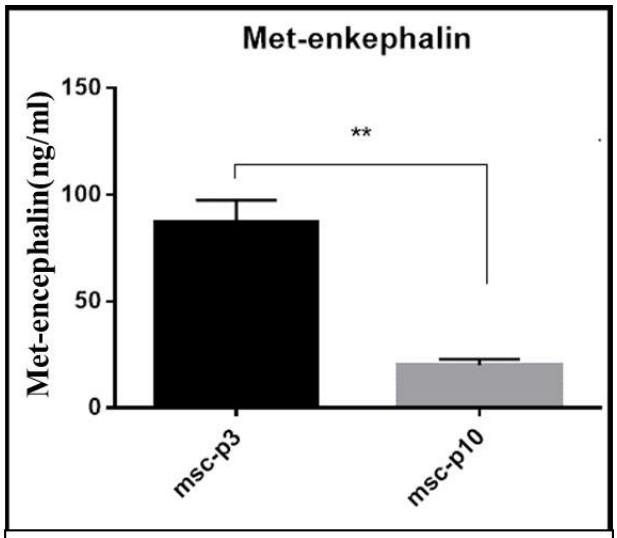

Fig. A1: Bar graphs showing that the levels of Met-encephalin released by hMSCs at p3 were significantly increased compared to those released by hMSCs at p10. Data were received with four independent experiments and presented as mean ± S.E.M. One-factor ANOVA was used to assess statistical significance between means. $^{**}P < 0.01$.

b. ELISA: hMSCs at passage 3 (p3) and passage 10 (p10) were respectively cultured at a concentration of 1×10^6 cells/well (6-well plate) in DMEM-L (Gibco) supplemented with penicillin 100 U/mL, streptomycin 100 U/mL, and 10% FBS. These cells were incubated at 37°C in a 5% CO2 humidified incubation chamber. Culture medium were refreshed twice a week. Following 3 day incubation, supernatant of cell culture was collected, centrifuged, and stored at -80°C before further analysis. The levels of Met-enkephalin in the supernatant of cell cultures were measured by an (enzyme-linked immunosorbent assay) ELISA kit (eBioscience, USA) according to the manufacturer's instructions. Each sample was performed in triplicates. Absorbance was read using a microplate reader set to 450 nm. Data were expressed as mean ± S.E.M. SPSS 16.0 was used to perform the statistical analysis. One-factor ANOVA was used to assess statistical significance between the means. The statistical significance was established at $p < 0.05$ ($*p < 0.05$, $**p < 0.01$). Results showed that both hMSCs at p3 and p10 can spontaneously secret Met-enkephalin in culture, however, the levels of Met-enkephalin released by hMSCs at p3 were significantly higher than those released by hMSCs at p10, with about 3-fold increases (Fig. A1). These data suggest that hMSCs with less passages may serve as a better suitable cell source than repeated passages (for example >p10) for potential pain management as a cell therapy.

2: Determine the analgesic and anti-tolerance effects and the safety of chromaffin-like cells in rats:

As reported above, we have successfully generated hCLCs *in vitro* by reprogramming hMSCs with the cellular extracts of porcine chromaffin cells. More importantly, intrathecal injection of these chromaffin-like cells in rats produced significant antinociceptive effect *in vivo* for responses to both Aδ and C thermal stimuli without using immunosuppressants (Fig. A, $p<0.01$). Our results suggest that analgesic CLCs can be produced from an individual's own tissue-derived MSCs by targeted cell reprogramming for the application of chronic pain management (24). Transplantation of cells derived from individuals' autologous tissue into the same individuals would be safe and immunocompatible compared to xeno- and allo-transplants. In practice, hMSCs can be derived from a patient's own tissues, easily cultured and expanded, and could not only be prepared to become isogeneic through cell reprogramming approach, but also could avoid immunological rejection through autologous cell transplantation. Thus, autologous hCLCs could be the most desirable alternative to real adrenal chromaffin cells for potential therapeutic purposes in clinics. Robust and long-lasting analgesic effects are expected for autologous hCLCs because these cells would be spared from immune responses, thereby improving the therapeutic efficacy of the

transplanted cells. Naïve rMSCs, hMSCs, and the generated hCLCs were shipped to Dr. Cheng (Initiating PI) lab at the Cleveland Clinic, where transplantation studies were conducted to investigate the analgesic and anti-tolerance effects of these cells in a rat model of neuopathic pain. Very promising positive data were produced (Fig. B). Fig. B showed that even naïve rMSCs (p<3) can produce important analgesic and anti-tolerance effects in the rats with cell transplantion. Experimental data have been published (44).

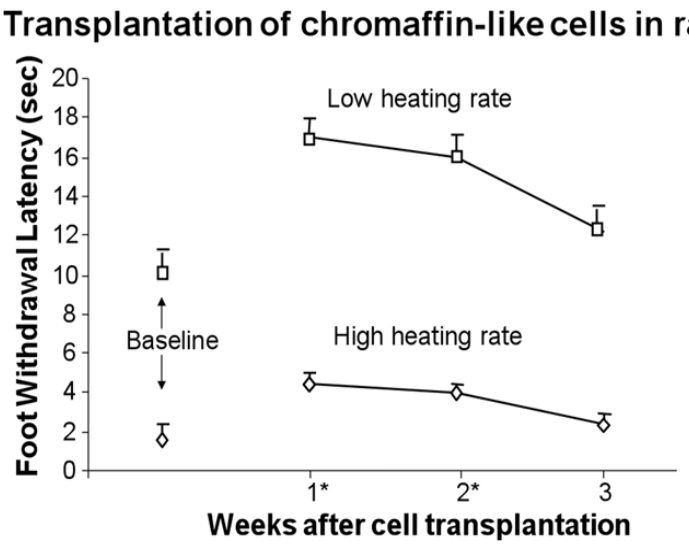

Fig. A. Effects of intrathecal transplantation of a single dose 1x10^5) of chromaffin-like cells on foot withdrawal latencies evoked by low (C nociceptor: 0.9°C/s, squares) and high (Ad nociceptor: 6.5°C/s, diamonds) heating rates on the dorsal surface of the feet in rats (n = 6). Foot withdrawal latencies were measured before cell transplantation (baseline) and remeasured 1 week and then weekly at 1-week intervals following cell transplantation. Data from baseline and response latencies at different time points after cell transplantation were expressed as the mean ± SEM and compared using t tests. Follow-up analysis was performed by Bonferroni post hoc tests. Results showed that transplantation of chromaffin-like cells increased foot withdrawal latencies evoked by both high and low heating rates for at least 3 weeks, with significant increases compared to baseline for 2 weeks (*p < 0.01, t tests and Bonferroni correction).

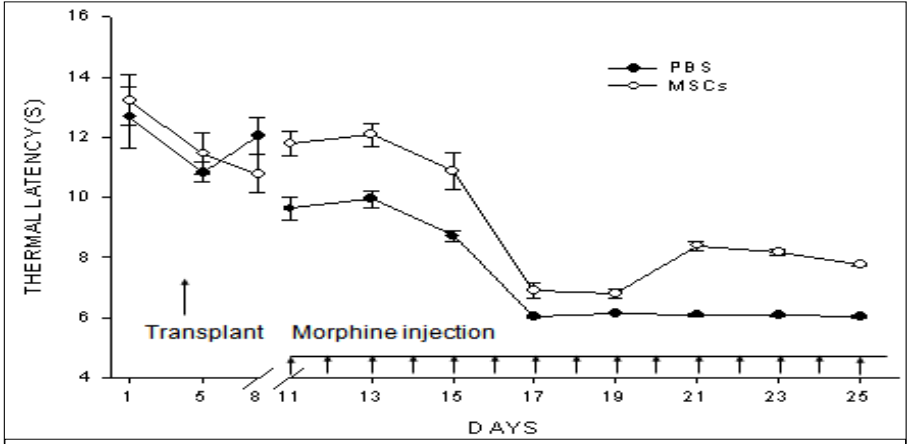

Fig B: Analgesic and anti-tolerance effects of rMSC (P<3) transplantation determined by the withdrawal thresholds (mean + sem) to thermal stimulation. Daily morphine injections significantly reduced the withdrawal thresholds in the rMSC group. However, the withdrawal thresholds were consistently higher in rMSC group than the control group throughout the whole course of daily morphine injection. Also, the withdrawal thresholds in days 21, 23, and 25 were also statistically higher than those in days 17 and 19, indicating an anti-tolerance effect of rMSC transplantation.

<u>2a. Generate neuropathic pain model and perform transplantation experiments; Perform analgesic evaluation; CSF sampling and measuring concentrations of enkephalins and catecholamines:</u>

The accomplishment for Task 2a was performed and completed at the Cleveland Clinic. Detailed experimental results were reported separately by Dr. Cheng (Initiating PI).

<u>2b. Determine cell dose response curve.</u>

Task 2b was reported separately by Dr. Cheng (Initiating PI).

2c. Determine anti-tolerance effects of autologous CLCs to repeated morphine administrations:

Previous studies demonstrated that transplantation of adrenal chromaffin cells in humans can play an important role in the analgesia and the inhibition of opioid tolerance, which may be attributed to a synergistic action of endogenous molecules released by these cells (1-8). MSCs and/or MSCs-derived CLCs may also produce such analgesic and anti-tolerance effects to chronic opioids. In the extended studies to explore the potential mechanism(s) of anti-tolerance effects of MSCs, we investigated the potential effect of MSCs to the development of morphine-induced tolerance *in vitro* by a co-culture system of MSCs and the neuronally-differentiated SH-SY5Y cells and *in vivo* by spinal transplantation of MSCs in rat pain models.

Neuronal differentiation of SH-SY5Y cells:

It is well known that the neuronally-differentiated SH-SY5Y cells, a well-established stable human neuroblastoma cell line, can be used as a cell model *in vitro* for neuroscience research. Retinoic acid (RA) is the most commonly used inducer for neuronal differentiation of SH-SY5Y cells. However, neuronal production is very low under RA treatment only combination of RA and other chemicals. Thus, we explored a new method to increase neuronal production of SH-SY5Y cells by using conditioned medium of human neural stem cells (CM-hNSCs) in cultures as neuronal medium.

SH-SY5Y cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and Sigma. These cells were cultured in the DMEM/F12 (Invitrogen), including 15% FBS, 2 mM L-glutamine, and an antibiotic–antimycotic mixture (1:100; Invitrogen) at 37°C in a 5% CO2 humidified incubation chamber. SH-SY5Y cells (1×10^5) were plated onto poly-lysine coated coverslips (diameter 10mm) in 1ml culture medium and divided into four groups: treated with 10μM RA (retinoic acid, Sigma) in DMEM/F12 (Invitrogen) including 3% FBS for 3 days (A) or 7 days (B), and treated with 10μM RA (retinoic acid, Sigma) in CM-hNSCs for 3 days (C) or 7 days (D). The fresh differentiation mediums were changed every day. Each experiment was performed in duplicate. As shown by Fig. C, our new method can effectively induce neuronal differentiation of SH-SY5Y cells and increase the proportion of neurons with 3 and 7 day treatment of CM-hNSCs and RA compared to that with only RA treatment, in which about 90% of differentiated cells showing positive beta-III tubulin staining, a well-accepted neuronal marker. In addition, maturation of the neurons differentiated from SH-SY5Y cells following CM-hNSCs and RA treatment was greatly improved, with long and multiple cell process as well as neural fiber development. It is the first study to use CM-hNSCs for promoting neuronal differentiation of RA treated SH-SY5Y cells. Detailed experimental results have been published (43). We are the first to report the use of CM-hNSCs in combination with RA can rapidly and effectively promote the neuronal production of RA-treated SH-SY5Y cells in culture conditions.

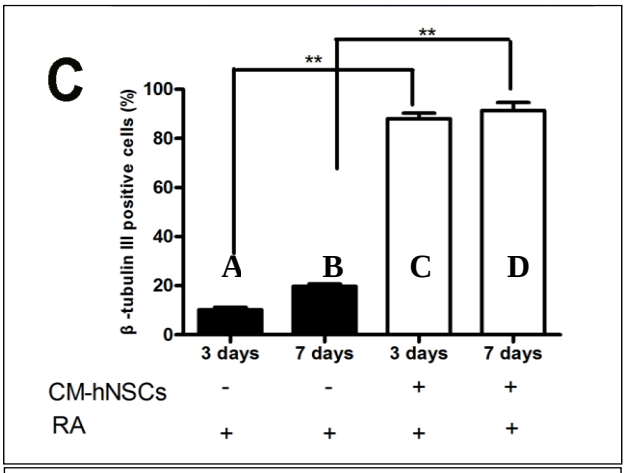

Fig. C: The bar graph showing that combinational induction of RA and CM-hNSCs significantly increased the proportion of β–tubulin III positive cells compared to those with RA treated alone. One way ANOVA, **P<0.01

Co-cultures of neuronally-differentiated SH-SY5Y cells with hMSCs or HDFs:

Before co-cultures, neuronally-differentiated SH-SY5Y cells (3 days after culturing CM-hNSCs and RA) were treated with morphine (10μM) for 24 h unless otherwise specified. Human bone marrow samples (n=4, three males, ages 22, 25, and 32; one female, age 50) were purchased from AllCells LLC (Emeryville, CA). The methods for isolating and culturing BM-derived hMSCs is the same as we previously described (20-24). In our designed experiments, hMSCs ≤passage 5 were defined as early passage, while hMSCs >passage 10 were defined as the late passage. Co-cultures of SH-SY5Y cells and hMSCs were performed by using the cell culture insert with 0.4μm diameter pores according to the manufacturer's protocol (Greiner Bio-One, Monroe, NC). Briefly, 100μl cell suspension containing different concentrations of hMSCs was pipetted onto the membrane of the cell culture insert (Fig. 1a) or the membrane of the inverted cell culture insert (Fig1. b) and the cells were allowed to adhere overnight at 37°C in a 5% CO_2 incubator. Subsequently, the cell culture insert was placed into the well of a 12 well plate pre-seeded with differentiated SH-SY5Y cells, which were pretreated with 10μM morphine for 24h. Co-cultures of SH-SY5Y cells and hMSCs were maintained for additional 36 hours in the presence of 10μM morphine at 37°C in a 5% CO_2 incubator. The primary human dermal fibroblasts (HDFs) were purchased from Cell Applications, Inc. (San Diego, CA) and cultured in 1640 medium containing 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The co-cultures of SH-SY5Y cells and HDFs of early passages (P≤5) were performed by using the same method described for co-cultures of SH-SY5Y cells and hMSCs at identical condition, as shown in Fig. 1a & b.

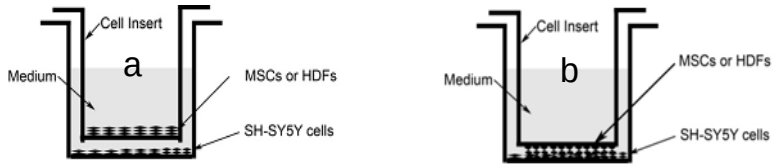

Fig. 1. Diagram of co-culture of MSCs or HDFs/SH-SY5Y cells to show (a) without cell contact, (b) with cell-cell contact.

cAMP accumulation assay:

cAMP levels, which is involved in opioid tolerance development as one of the cellular mechanisms, was determined in the morphine-treated SH-SY5Y cells. Thirty-six hours after co-cultured with hMSCs or HDFs, SH-SY5Y cells were harvested with a non-enzymatic cell dissociation solution followed by washing once with HBSS buffer. cAMP accumulation in SH-SY5Y cells was assayed by using a LANCE™ cAMP kit (PerkinElmer, Waltham, MA) according to the manufacturer's protocol. SH-SY5Y cells were centrifuged at 1000g for 2 min and re-suspended at a concentration of 2×10^6 cells/ml in stimulation buffer (1×HBSS, 5 mM HEPES, 0.1% BSA, 0.5 mM IBMX, pH 7.4) and mixed with 50μM forskolin. The Alexa pluor®647 labeled antibody was added to the cell suspension and the cells were incubated at 37 °C for additional 15 min, the detection mix was then added to the final cell suspension. The cell sample was further incubated for 1h in the dark and read on a TECAN instrument (San Jose, CA) to measure the LANCE signal. The LANCE signal obtained at 665 nM was directly used to analyze the cAMP levels. The signal at 615nM was used to identify dispensing or quenching problems. The cAMP standard curve was assayed according to the manufacturer's instructions. The cAMP formation was calculated as the percentage of forskolin-stimulated cAMP accumulation without morphine, which was defined as 100% in our experiments. All data were expressed as Mean±SD. Statistical comparisons were analyzed using Prism software. Values of $p<0.05$ were considered statistically significant.

Immuocytochemistry:

Fluorescence immunocytochemical staining was performed on cultured neuronal cells differentiated from SH-SY5Y cells seeded on coverslips. SH-SY5Y cells without co-culture served as controls. Cells were washed with PBS and fixed in 4% paraformaldehyde (Sigma) in PBS (pH 7.4) for 20 min at room

temperature. Following washing in PBS, cell samples were blocked in PBS buffer containing 0.1% Triton X-100 (Sigma-Aldrich) and 3% donkey serum (Jackson ImmunoResearch) for 30 min, followed by incubation with rabbit anti-opioid receptor (MOR), (1:1000, ImmunoStar, Inc, Hudson, WI) and mouse anti-Rab5, a marker of early endosomes, (1:1000, Abcam Inc., Cambridge, MA) antibodies overnight at 4°C. Then the cells were washed in PBS and incubated with corresponding secondary antibodies, including rhodamine (TRITC)-conjugated or fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch) and donkey anti-mouse IgG antibodies for 2 h at room temperature in the dark. Finally, the cells were washed with PBS, counterstained with DAPI (Vector Labs, Burlingame, CA, USA), and viewed fluorescence microscopy (Zeiss, Jena, Germany). In total, three independent experiments were performed, and the cells double-immunostained by both antibodies were recorded.

Results:

1. MSCs inhibited the development of morphine-induced tolerance in SH-SY5Y cell model.

Exposure of 1 and 10μM morphine for 24h led to significantly upregulated cAMP levels in cultured SH-SY5Y cells when compared to the group without morphine treatment (Fig.2, $p<0.05$~$p<0.001$). The cAMP levels were increased to 150.7±20.6 by 1μM morphine and 241.6±19.6 by 10μM morphine, respectively, as shown in Figure 2. The cAMP levels of SH-SY5Y cells were then tested at different time points (0~48h) when treated by 10μM morphine, as shown in Figure 3, Treatment of SH-SY5Y cells with morphine caused significant increase of cAMP concentration in SH-SY5Y cells at 24 and 48h (Fig. 3, $p<0.001$). The levels of cAMP upregulated in the treated SH-SY5Y cells by morphine resulted in ~2.5 fold increase compared to that of SH-SY5Y cells without morphine treatment (Fig. 3). These results are consistent with the previous study (27) and show that SH-SY5Y cells are an appropriate cellular model for investigating the tolerance induced by chronic morphine treatment. Fig. 4 showed that following pretreatment with 10μM morphine for 24h, SH-SY5Y cells were co-cultured with MSCs or HDFs for additional 36h. Results revealed that the upregulation of cAMP induced by morphine in SH-SY5Y cells was significantly attenuated by hMSCs of early passage (≤P5), with $p<0.05$ at a ratio of 1/25 for hMSCs/SH-SY5Y cells and $p<0.01$ at a ratio of 1/5 for hMSCs/SH-SY5Y cells by cell-to-cell contact in the co-cultures, demonstrating a tendency of enhanced inhibition to the development of

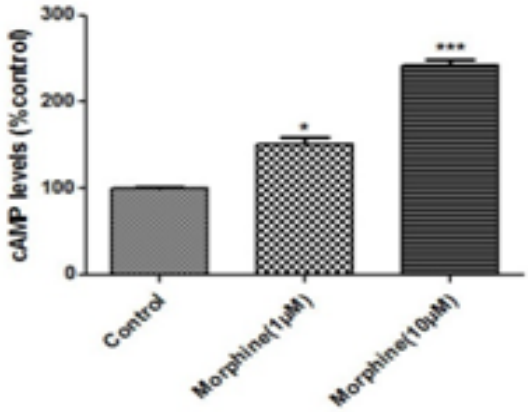

Figure 2: The effect of morphine on cAMP levels in SH-SY5Y cells: SH-SY5Y cells were pretreated with morphine for 24h, and the cAMP levels in SH-SY5Y cells were measured. The cell samples without morphine treatment served as a controls. * p<0.05, *** p<0.001. Data were presented as the Mean ± SEM of three independent experiments.

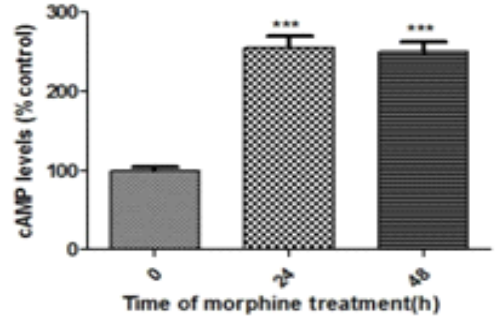

Figure 3: Time course of the development of cAMP upregulation in SH-SY5Y cells: SH-SY5Y cells were incubated with the medium containing 10μM morphine for 48h, cAMP levels were measured at different time points. The cell samples without morphine treatment served as a controls. *** p<0.001. Data represent Mean ± SEM of three independent experiments.

morphine tolerance in SH-SY5Y cells with the increasing ratio of hMSCs in co-cultures (Fig. 4A). HDFs (≤P5) and hMSCs of late passages (P>10) did not show any detectable inhibitory effect to the morphine-induced cAMP upregulation (Fig. 4A). HDFs are widely considered to be terminally differentiated and

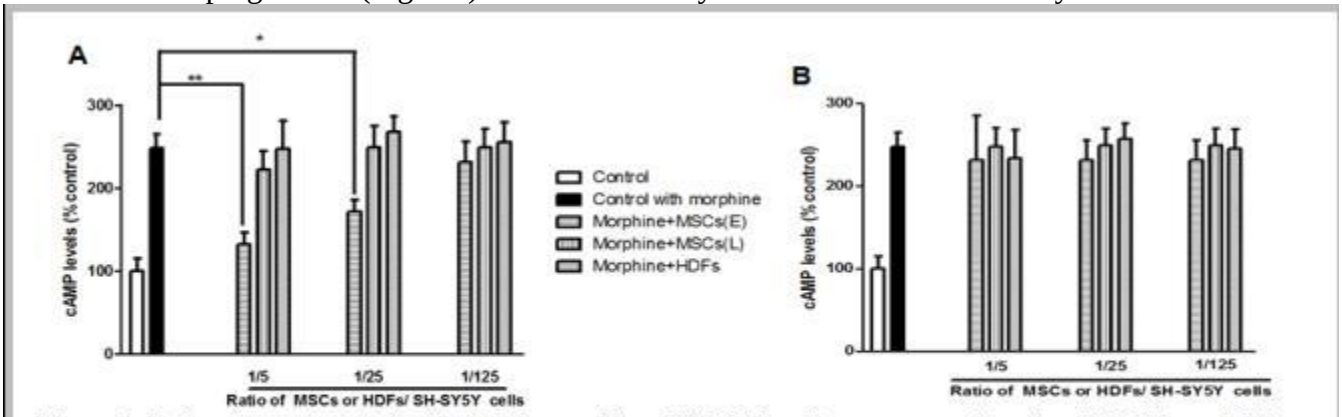

Figure 4: Following pretreated with 10μM morphine, SH-SY5Y cells were co-cultured with MSCs or HDFs with cell-cell contact (a) and without cell contact (b) for 36h at various ratio (1:5, 1:25, 1:125) in a high glucose DMEM medium containing 3% FBS and 10μM morphine and the cAMP levels of SH-SY5Y cells were then assayed. SH-SY5Y cells cultured alone with and without morphine treatment served as controls. Data were presented as the Mean ± SEM of three independent experiments. * p<0.05, ** p<0.01.

lack differentiation and colony-forming potential (34). Our results suggest that the anti-tolerance effect is specific to hMSCs and may be attributed to the interaction of cell-to-cell contact in the co-cultures between SH-SY5Y cells and hMSCs since no such effect was detected in the co-cultures of the same cells without cell physical contact (Fig. 4B). In addition, the proliferation rate of hMSCs of early passages, one of the characteristics of stem cells, is significantly higher than that of hMSCs of late passages (P>10, data not shown), and hMSCs of late passages may have differentiated or gradually lost their "stemness" during long period of *in vitro* culture, suggesting that the "stemness" of hMSCs at the early stage may play a crucial role in the anti-tolerance effect observed in our studies. Thus, further studies are warranted to clarify the mechanism(s) of anti-tolerance effect of hMSCs.

Opioid therapy is the cornerstone of pain management (28, 29, 30). However, the long-term use of opioids for the pain management is hampered by analgesic tolerance, which requires escalating doses of drug to maintain pain relief at the same level. Other negative health consequences related with the long term use of opioids include cognitive impairment, drug abuse, and addiction. Although the molecular mechanisms of opioid tolerance are still unclear, the most observed correlative biochemical adaptation both *in vitro* and *in vivo* is the upregulation of cyclic adenosine monophosphate (cAMP) system, including increased intracellular cAMP levels, adenylate cyclase supersensitization, and other chronic changes that involve activation of transcription factors leading to alterations in protein expression (31, 32, 33). Thus, cAMP upregulation after chronic opioids has been proposed as a biochemical mediator underlying chronic effects of opioids and a cellular hallmark to study morphine tolerance. In our experiments to generate analgesic and anti-tolerant CLCs from hMSCs, we found that naïve hMSCs at the early passages (≤passage 5) showed significant inhibitory effect on the development of morphine-induced tolerance in differentiated SH-SY5Y cells, i.e., significantly attenuated morphine-induced cAMP up-regulation (Fig. 4A), suggesting hMSCs may have therapeutic potential for the opioid tolerance treatment in clinics.

2. MSCs increased the expression and co-localization of MOR and Rab 5 in morphine-treated SH-SY5Y-cells.

Immunocytochemical examination of morphine-treated SH-SY5Y cells showed a re-distribuation of mu opioid receptor (MOR) and augmented expression of Rab5, a marker of early endosomes, as well as increased co-localization of MOR and Rab5 when co-cultured with hMSCs of early passages (≤passage 5, Fig. 5). An important mechanism of opioid receptor regulation involves endocytosis of receptors, failure of MOR endocytosis contributes to the development of tolerance and dependence of opioids (35, 36). In SH-SY5Y cellular model, we have shown that hMSCs can inhibit cAMP upregulation induced by chronic morphine, a hall mark of tolerance and dependence, the interesting immunocytochemical results of our studies, i.e., the enhanced activities of MOR and Rab5 of SH-SY5Y cells by hMSC co-culture may be also involved in the reduction of morphine tolerance development in these cells. Thus, we propose the notion that application of hMSCs may promote the activities of MOR and Rab of neuronal cells to opioids, thereby preventing its tolerance development (23).

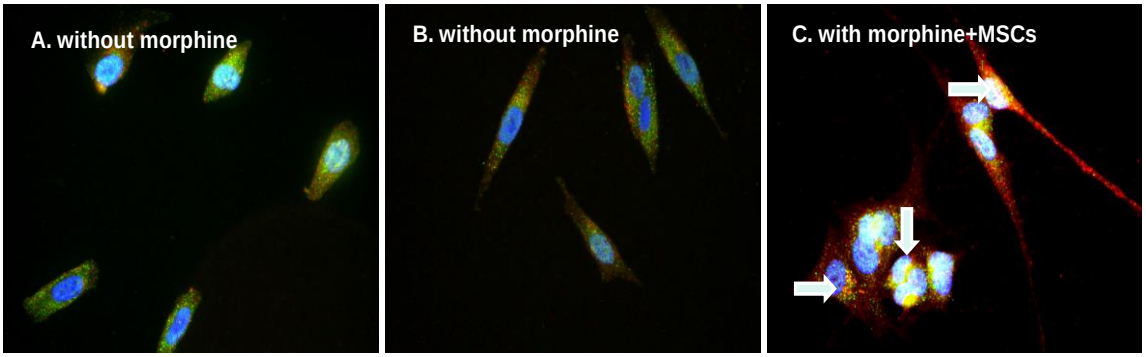

Figure 5. Representative immunocytochemical staining pictures of MOR and Rab 5 in morphine-treatedSH-SY5Y cells processed for fluorescence microscope. In SH-SY5Y cells without (A) and with (B) morphine, MOR (green) and Rab 5 (red) were distributed in the cytoplasm with less Rab 5 immuno staining. In morphine-treated SH-SY5Y cells with hMSC co-culture (C), MOR expression was re-distributed toward the intracellular compartment close to the nuclei with increased co-localization with strong Rab 5 immunostaining (yellow, arrows indicated), suggesting an enhanced activities of MOR and Rab 5 in these cells.

3. MSCs produced significant analgesic and robust anti-tolerance effects in rat pain models:

The analgesic and anti-tolerance effects of rMSCs at the early stage (passages 2-3) are confirmed in animal pain models. Details were reported separately by Dr. Cheng with a recent publication (44).

2d. Determine the safety of chromaffin-like cell therapy: Determine the fate of the transplanted cells and their phenotypic stability:

Based on the current data, the animals with hCLC, hMSC, and rMSC transplantation maintained normal food and water intake, locomotor function, and body weight, no remarkable adverse effect was observed, suggesting the safety of these cells as transplants. Postmortem spinal cord samples in total with rMSC (4) and vehicle (4) injections have been received from Dr. Cheng's lab and immunohistological examination was conducted to locate the transplanted cells and determine the cell fate, phenotypic stability of the transplanted cells, and potential inflammatory responses to the transplants were performed.

Immunohistochemistry: The lumber segment of the spinal cords covering the site of cell injection was post-fixed overnight and then transferred to a 30% sucrose buffer solution. Sagittal sections (5-20 µm) were cut on a cryostat. Fluorescence immunohistochemical staining was performed on these sections. Following washing in PBS, sections were blocked in PBS buffer containing 0.1% Triton X-100 (Sigma-Aldrich) and 3% donkey serum (Jackson ImmunoResearch) for 30 min, followed by incubation with antibodies overnight at 4°C, including: mouse anti-TH (1:300; Sigma-Aldrich), rabbit anti-Met-enkephalin (1:600; ImmunoStar Inc., Hudson, WI), specific markers for chromaffin cells, mouse anti-fibronectin (1:2000, Sigma), a marker for MSCs, and mouse anti CD11b (1:600, Abcam Inc., Cambridge, MA), a marker for microglial cells. Sections were then washed in PBS and incubated with corresponding

secondary antibodies, including fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG, and rhodamine (TRITC)-conjugated donkey anti-rabbit IgG antibodies (1:200; Jackson ImmunoResearch) for 2 h at room temperature in the dark. Finally, the sections were washed with PBS, counterstained with DAPI (Vector Labs, Burlingame, CA), and viewed with immunofluorescence microscopy (Zeiss, Jena, Germany). Fibronectin for MSCs and markers for chromaffin cells were not detected in the examined spinal sections (data not shown); suggesting that cells injected into the intrathecal or subarachnoid space of spinal cord may have floated into the cerebrospinal fluid (CSF) from the injection site. Other important immuno staining for inflammatory cells are reported by Dr. Cheng lab.

3: Determine the safety of CLCs in SCID mice:

Task 3 is completed. Experiments was designed to rule out the possibility of tumor formation from human CLCs (hCLCs) transplanted to severe combined immunodeficient (SCID) mice. In total, 24 NOD.CB17PrkdcSCID/J mice (2.5± month old, 30g±) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a specific pathogen-free environment throughout the experiments.

Injection of hCLC in SCID mice: Under anesthesia with ketamine (100mg/kg) and xylazine (10mg/kg, i.p.), hCLCs was injected into the spinal cord lumbar subarachnoid space (n=12) or tail vein (i.v., n=12), respectively. For spinal injection, the hair of injection site was removed by electric razor, an iodine swab was applied to the skin around the injection area of the L4-L5 lumbar interspace. The iliac crest of mouse was provide a rough estimate for this interspace level for cell injection. A 29 G spinal needle was used and inserted through skin into the subarachnoid space. A single dose of hCLCs ($1x10^5$ in 5µl culture solution) was slowly injected into the subarachnoid space through the L4–L5 lumbar interspace using a Hamilton microsyringe. The injection was delivered for 5 minutes and the needle was left in place for an additional 2 min following the injection before carefully withdrawal. For tail vein injection, iodine swab was used to disinfect the tail skin before injection, then a single dose of CLCs ($1x10^5$ in 5µl culture solution) was injected slowly into the tail vein with a 25G needle syringe, followed by a 2 min delay and light pressure to skin of the injection site before withdrawal of the needle.

Observation: After cell transplantation, no tumor formation was noted on gross observation in SCID mice during the observation periods for up to about 6 months. No immediate or delayed toxic reactions including allergic reactions associated with hCLC transplantation were detected. No other significant complications associated with the hCLC transplantation observed in these mice.

Perfusion: At 3 and about 6 months (5 month 20 days), respectively, after cell injections, mice (n=6 from each group) were anesthetized with ketamine/xylazine (0.1 ml/10g BW), the heart was exposed, a needle connected to a plastic pipe was inserted to left ventricle followed by right atrium cut. Perfusion was performed through the heart with PBS (50ml) and then followed by 4% paraformaldehyde (PAF) in PBS (100ml). Spinal cord, brain, and other major organs including lung, heart, liver, kidney, and spleen were removed for more detailed examination. No enlarged lymph node was detected. Subsequent immunohistochemical examination was conducted for potential presence of abnormal growth of the transplanted cells.

Immunohistochemical examination: The removed organs and tissues were post-fixed overnight in 4% PAF in PBS and then transferred to a 30% sucrose in PBS containing 4% PAF for additional 1-2 days. Sections (30µm) were cut on a cryostat. Fluorescence immunocytochemical staining was performed on sliced sections. In brief, following washing in PBS, sections were blocked in PBS buffer containing 0.2% Triton X-100 (Sigma-Aldrich) and 3% donkey serum (Jackson ImmunoResearch) for 30 min, followed by incubation with primary antibodies overnight at 4°C, including: mouse monoclonal anti-human nuclei (1:100; Chemicon), which specifically labels human cells, and rabbit polyclonal anti-fibronectin (1:600;

Abcom), one of the markers for hMSCs. Positive controls for specific human nucleus staining were conducted in the brain of mice at 1-2 days after receiving nasal hMSC infusion. Negative controls for nonspecific staining were conducted with the same protocol without primary antibodies. Sections were then washed in PBS and incubated with corresponding secondary antibodies, including fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (green) and rhodamine (TRITC)-conjugated donkey anti-rabbit IgG antibodies (red) or vice versa (1:200; Jackson ImmunoResearch) for 2 h at room temperature in the dark. Finally, the sections were washed with PBS, counterstained with DAPI (blue, Vector Labs, Burlingame, CA), and viewed with immunofluorescence microscopy (Zeiss, Jena, Germany). Digital images were captured by fluorescent microscopy and analyzed by NIH Image software (NIH).

Results and Discussion: In the present study, we injected 24 mice with hCLCs and none developed tumors during the observation period for up to about 6 months. The injected hCLCs were not detected in the sections examined by fluorescence immunostaining since specific human nucleus staining did not localize any positive cells or nuclei. Additionally, although the antibody for fibronectin we used in the immunohistochemical examination can recognize fibronectin of both human and rodent origins, we did

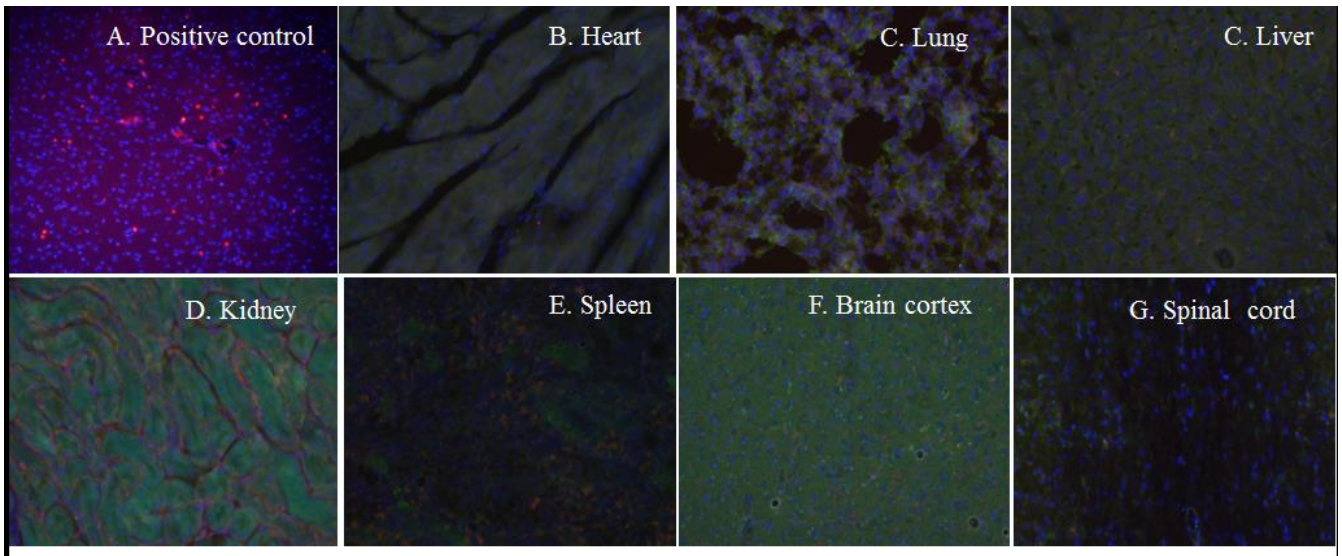

Fig 6. A. Positive control of the human nucleus antibody, showing intense nuclear immunostaining in the mouse brain at 1-2 days following nasal infusion of hMSCs. Red: specific staining for human cell nucleus, Blue (DAPI): staining for all of the cell nuclei. No human cell nucleus staining was detectable in all of the tissue sections including B. heart, C. lung, D. liver, E. kidney, F. spleen, G. brain, and F. spinal cord from SCID mice with spinal and systemical injection of hCLCs.

not find any co-localization or double-labelling of fibronectin and human nucleus in the examined tissues of SCID mice with hCLC transplants. That is, by using specific human nucleus antibody, we excluded any abnormal growth of cells with human origin in SCID mice. Other immuno staining was not performed after confirming that the applied hCLCs were not detectable in these mouse tissue. For spinal cord injected mice, we did not find any detectable human cells in spinal cord and brain tissues. As discussed previously in rat pain models, cells injected into the subarachnoid space of spinal cord may have floated into CSF from the injection site and become undetectable. Although systemically (i.v.) injected MSCs can be found to accumulate in the lungs, followed by in liver and spleen in the early stage of administration (37), i.v. injected hCLC were not observed in these organs after careful examination. As reported previously, i.v. injected MSCs are often not detectable at all at later time points, or only a small fraction of them can be traced (38-42). Consistent with these studies by others, we did not localize those i.v. injected hCLCs or hMSCs in all of the examined tissues, including heart, liver, lung, kidney, spleen, brain, and spinal cord (Fig. 6). Since many important therapeutic effects have been observed without detectable MSCs in the target tissues, further studies are warranted to clarify the bio-distribution,

migration, and final fate or elimination of systemically applied MSCs. Our current data provide evidence to support that hCLCs and their parent cells, i.e., hMSCs, may be safe candidates for potential therapeutic applications in humans.

4. Key Research Accomplishments:

- A new finding that hMSCs at the early passages (<passage 5) resulted in a significant inhibition on the development of morphine tolerance in neuronally differentiated SH-SY5Y cells in the co-culture, with a down-regulation of morphine-induced cAMP production as well as augmented expression of Rab5 and increased co-localization of Rab5 and MOR in SH-SY5Ycells-differentiated neurons (23).

- A new finding that spinal transplantation of rMSCs at the early passages ($p \leq 3$) produced significant analgesic and robust anti-tolerance effects in an animal pain model.

- No tumor formation and abnormal cell growth from the transplanted hCLCs were found in SCID mice.

5. Conclusion:

Our research is conducted as planned in the approved SOW and have confirmed that CLCs generated from hMSCs via cell reprogramming have a significant analgesic effect in rat models. Our new findings as listed above in the "Key Research Accomplishments" that even naïve MSCs in their early passages (<passage 5) without cell reprogramming can also result in significant inhibition of pain and morphine tolerance (44) will have a substantive impact on the final goals of this DOD grant, suggesting that MSCs may serve as valuable and effective therapeutic cells for treating chronic pain and analgesic tolerance, with a potential to reduce the need for exogenous opioid medications and minimize the risk of prescription drug abuse and addiction. In addition, when transplanted, no tumor formation and abnormal cell growth of hCLCs were found in SCID mice, suggesting the safety of these cells as therapeutics. Our confidence is further supported by the recent findings from other independent investigators that MSCs not only can reduce pain but also inhibit activation of microglia and astrocytes which play a critical role in the development and maintenance of chronic pain (28, 29). In the future, we will compare the therapeutic values of these early passaged naïve MSCs and the reprogrammed MSCs (CLCs), including their analgesic and anti-tolerance effects, to expand our research and determine the best cell types for clinical application. We will also compare intravenous vs. intrathecal application of MSCs to determine the best route of clinical use. In this way, we hope to apply the most easily-handled cell therapies with long-lasting analgesic and anti-tolerance effects for treating patients with a wide range of pain states to improve quality of life and save billions of dollars for the military health and disability systems.

6. Publications, Abstracts, and Presentations:

Publications:

Qu T*, Shi GB, Ma K, Yang H, Duan WM, and Pappas GD., Targeted cell reprogramming produces autologous analgesic cells from human bone marrow-derived mesenchymal stem cells, Cell Transplant., 2013, 22 (12):2257-2266

Yang H, Wang J, Sun J, Liu X, Duan WM, **Qu T*.** A new method to effectively and rapidly generate neurons from SH-SY5Y cells. Neurosci Lett. 2016 Jan 1;610:43-7.

Hua Z, Liu L, Shen J, Cheng K, Liu A, Yang J, Wang L, **Qu T,** Yang H, Li Y, Wu H, Narouze J, Yin Y, Cheng J. Mesenchymal Stem Cells Reversed Morphine Tolerance and Opioid-induced Hyperalgesia. Sci Rep. 2016 Aug 24;6:32096. PMID: 27554341

Abstracts:

Shi GB, Ma K, Pappas GD., and **Qu T*,** Targeted cell reprogramming produces autologous analgesic cells from human bone marrow-derived mesenchymal stem cells, Military Health System Research Symposium (MHSRS), Harbor Beach Marriott, Ft Lauderdale, Florida, August 13-16, 2012

Sun JH, Yang HN, and **Qu T*,** Human mesenchymal stem cells from bone marrow attenuate morphine tolerance *in vitro*, The 3rd Annual Research Forum-Extravaganza, Department of Psychiatry, University of Illinois at Chicago, Chicago, IL, September 19, 2012

Yang HN, Sun JH, Wang F, and **Qu T***, Attenuation of chronic morphine-mediated cAMP upregulation in SH-SY5Y cells by mesenchymal stem cells, The 2nd International Conference and Exhibition on Addict ion Research & Therapy, Las Vegas, USA, July 22-24, 2013.

Hongna Yang, Yan Li, Feng Wang, and **Tingyu Qu***, A new method to generate neurons effectively from cultured SH-SY5Y cells. The 5th Annual Research Forum Extravaganza, Department of Psychiatry, University of Illinois at Chicago, Chicago, IL, Oct. 21, 2014

Yan Li, Feng Wang, HongNa Yang, and **Tingyu Qu***, Mesenchymal stem cells inhibit morphine-mediated tolerance development, The 5th Annual Research Forum-Extravaganza, Department of Psychiatry, University of Illinois at Chicago, Chicago, IL, Oct. 21, 2014.

7. Inventions, Patents, and Licenses:

Patents:

Qu T*, Cheng J, Sun J, and Yang HN, 2012/DG020, Human mesenchymal stem cells attenuate morphine tolerance – Provisional Patent Application for Technology DG020/2013-020 (Office of Technology Management (OTM) of UIC)

Qu Tingyu*, Cheng Jianguo, Sun Jinhua, Yang Hongna, Cheng Kathleen, Shen Jun, Methods Of Attenuating Opioid Tolerance, Sep 3, US patent 2015/0246074 A1

8. Reportable Outcomes:

Autologous naïve hMSCs at may serve as cellular therapeutics in chronic pain management with great potential to reduce pain and inhibit tolerance development.

A patent "Methods Of Attenuating Opioid Tolerance (US patent 2015/0246074 A1) " was produced and published.

9. Other Achievements:

1) Dr. Tingyu Qu (Partnering PI) was promoted from Assistant Research Professor to Associated Research Professor in the Department of Psychiatry and the Department of Anatomy and Cell Biology at UIC in 2014 and continuously received funding from the Boothroyd Foundation for stem cell research.

2) HongNa Yang, PhD student, graduated and received PhD degree in the Medical School of Shandong University, China in 2013. She is associated with two publications, one submitted manuscript, four presentations, and one patent application supported by this DoD grant.

3) Feng Wang, PhD student, graduated and received PhD degree in the Medical School of Shandong University, China in 2015. He is associated with one publication, one submitted manuscript, and three presentations supported by this DoD grant.

List of personnel receiving pay from the research effort:

Tingyu Qu, Research Associate Professor
JinHua Sun, Post-doctoral Associate
HongNa Yang, PhD student
Feng Wang, PhD student

Other Publications:

Yang H, Sun J, Wang F, Li Y, Bi J, **Qu T***. Umbilical cord-derived mesenchymal stem cells reversed the suppressive deficiency of T regulatory cells from peripheral blood of patients with multiple sclerosis in a co-culture - a preliminary study. Oncotarget. 2016 Sep 29. PMID: 27705922

Yang H, Sun J, Li Y, Duan WM, Bi J, **Qu T*,** Human umbilical cord-derived mesenchymal stem cells suppress proliferation of PHA-activated lymphocytes in vitro by inducing CD4+CD25highCD45RA+ regulatory T cell production and modulating cytokine secretion, Cell Immunol, 2016, 302:26-31

Yang H, Wang J, Wang F, Liu X, Chen H, Duan W, **Qu T***, Dopaminergic Neuronal Differentiation from the Forebrain-Derived Human Neural Stem Cells Induced in Cultures by Using a Combination of BMP-7 and Pramipexole with Growth Factors, Front Neural Circuits, 2016 Apr 20;10:29. PMID: 27147976

Li C, Zhang YX, Yang C, Hao F, Chen SS, Hao Q, Lu T, **Qu T**, Zhao LR, Duan WM, Intraventricular administration of endoneuraminidase-N facilitates ectopic migration of subventricular zone-derived neural progenitor cells into 6-OHDA lesioned striatum of mice. Exp Neurol. 2015, 24;277:139-149

Hao Q, An JQ, Hao F, Yang C, Lu T, **Qu T**, Zhao LR, Duan WM. Inducible Lentivirus-Mediated Expression of the Oct4 Gene Affects Multilineage Differentiation of Adult Human Bone Marrow-Derived Mesenchymal Stem Cells. Cell Reprogram. 2015, 17(5):347-59.

Yang HN, **Qu T**, Yang H, Wei LF, Xie ZH, Wang P, Bi JZ, Self-assembling nanofibers improve cognitive impairment in a transgenic mouse model of Alzheimer's disease, Neurosci Lett. 2013,27;556:63-8

Zhang LJ, Xue YQ, Yang C, Yang WH, Chen L, Zhang QJ, **Qu T,** Huang S, Zhao LR, XM Wang , Duan WM, Pre-immunization with an intramuscular injection of AAV9-human erythropoietin vectors reduces the vector-mediated transduction following re-administration in rat brain, PLoS ONE, 2013, May 8;8(5):e63876

Zhang LJ, Xue YQ, Yang C, Yang WH, Chen L, Zhang QJ, **Qu T,** Huang S, Zhao LR, XM Wang , Duan WM*, Human albumin protects nigral dopamine neurons against 6-hydroxydopamine neurotoxicity by suppressing activation of MAPK signaling, PLoS ONE, 7(7) e41226, 2012

10. References:

1. Bés JC, Tkaczuk J, Czech KA, Tafani M, Bastide R, Caratero C, Pappas GD, Lazorthes Y. One-year chromaffin cell allograft survival in cancer patients with chronic pain: morphological and functional evidence. Cell Transplant. 1998, 7(3):227-38.

2. Lazorthes, Y., Sagen, J., Sallerin, B., Tkaczuk, J., Duplan, H., Sol, J.C., Tafani, M., Bes, J.C., Human chromaffin cell graft into the CSF for cancer pain managemant: a prospective phase II clinical study, Pain 2000, 87:19-32.

3. Lazorthes Y, Bès JC, Sagen J, Tafani M, Tkaczuk J, Sallerin B, Nahri I, Verdié JC, Ohayon E, Caratero C, et al. Transplantation of human chromaffin cells for control of intractable cancer pain. Acta Neurochir Suppl. 1995, 64:97-100.

4. Pappas, G.D., Lazorthes, Y., Bes, J.C., Tafani, M., Winnie, A.P. Relief of intractable cancer pain by human chromaffin cell transplants: Experience at two medical centers, Neurological Research 1997, 19:71-77.

5. Pappas, G.D. (1998). Transplantation of chromaffin cells for treatment of chronic pain: clinical biochemical and morphological findings. In: The Adrenal Chromaffin Cells (Kanno, Nakazato and Kumakura, eds). Hokkaido University Press, Sapporo, Japan, pp. 343-350.

6. Lazorthes, Y., Bes, J-C., Sol, J-C., and Pappas, G.D. (2001) Intrathecal chromaffin cell allograft for cancer pain. In: Surgical Management of Pain, Burchiel, ed., Thieme Medical Publishers, Inc., New York.

7. Livett, B.G., Dean, D.M., Whelan, L.G., Udenfriend, S., Rossier, J. Co-release of enkephalin and catecholamines from cultured adrenal chromaffin cells. Nature, 1981, 289(5795):317-9.

8. Unsicker K. The trophic cocktail made by adrenal chromaffin cells. Exp Neurol. 1993, 123(2):167-73. Review.

9. Hama A, Sagen J. Alleviation of neuropathic pain symptoms by xenogeneic chromaffin cell grafts in the spinal subarachnoid space. Brain Res. 1994, 651:183–193.

10. Sagen, J., Pappas, G.D., Pollard, H.B., Analgesia induced by isolated bovine chromaffin cells implanted in rat spinal cord. Proc Natl Acad Sci U S A. 1986, 83(19), 7522-6.

11. Sagen, J., Kemmler, J.E., Wang, H., Adrenal medullary transplants increase spinal cord cerebrospinal fluid catecholamine levels and reduce pain sensitivity. J Neurochem. 1991, 56(2):623-7.

12. Lu, Y., Jing, R., Yeomans, D.C., Pappas, G.D., Porcine chromaffin cells, culture, and transplant for antinociceptive effects in rodents and primates. Neurol Res. 2004, 26(7):707-12.

13. Ortega, J.D., Sagen, J., Pappas, G.D., Short-term immunosuppression enhances longterm survival of bovine chromaffin cell xenografts in rat CNS. Cell Transplant. 1992, 1(1):33-41.

14. Sagen J, Pappas GD, Perlow MJ. Adrenal medullary tissue transplants in the rat spinal cord reduce pain sensitivity. Brain Res 1986, 384:189–194

15. Sol, J.C., Sallerin, B., Larrue, S., Li, R.Y., Jozan, S., Tortosa, F., Mascott, C., Carraoue, F., Tafani, M., Lazorthes, Y., Intrathecal xenogeneic chromaffin cell grafts reduce nociceptive behavior in a rodent tonic pain model. Exp Neurol. 2004, 186(2):198-211.

16. Collas, P., and Hakelien, A. M. Teaching cells new tricks. Trends Biotechnol. 2003, 21:354–361.

17. Hakelien, A. M., Gaustad, K. G., and Collas, P. Modulation of cell fate using nuclear and cytoplasmic extracts. Methods Mol. Biol. 2006, 325:99–114.

18. Hakelien, A. M., Gaustad, K. G., Taranger, C. K., Skalhegg, B. S., Kuntziger, T., and Collas, P. Long term in vitro, cell type specific genome wide reprogramming of gene expression. Exp. Cell Res. 2005, 309:32–47.

19. Landsverk HB., Hakelien AM., Kuntziger, T., et al., Reprogrammed gene expression in a somatic cell-free extract. EMBO Reports 2002, 384-389.

20. Shi, G., Ma, K., Qu, T., and Pappas GD. Reprogramming human mesenchymal stem cells to achieve the phenotypic characteristics of adrenal chromaffin cells by using cellular extracts and cell fusion. 37th annual meeting of Society for Neurosciences, San Diego, CA, Nov. 3-7, 2007.

21. Shi, G., Ma, K., Pappas, GD., and Qu, TY., Phenotypic characteristics of hybrid cells produced by cell fusion of porcine adrenal chromaffin cells with human mesenchymal stem cells: A preliminary study. Neurol Res., 2008, 30(3):217-22.

22. Shi, G., Ma, K..,Li, R., Pappas GD., Qu, T., Analgesic effects of chromaffin-like cells produced *in vitro* via cell reprogramming of human bone marrow-derived mesenchymal stem cells. 39th annual meeting of Society for Neurosciences, Chicago, IL, Oct. 17-21, 2009.

23. Yang HN, Sun JH, Wang F, and Qu T, Attenuation of chronic morphine-mediated cAMP upregulation in SH-SY5Y cells by mesenchymal stem cells, The 2nd International Conference and Exhibition on Addict ion Research & Therapy, Las Vegas, USA, July 22-24, 2013

24. Qu T, Shi GB, Ma K, Yang H, Duan WM, and Pappas GD., Targeted cell reprogramming produces analgesic chromaffin-like cells from human bone marrow-derived mesenchymal stem cells, Cell Transplant., 2013, 22 (12):2257-2266

25. He L, Whistler JL, An opiate cocktail that reduces morphine tolerance and dependence. Curr Biol, 2005, 15: 1028–1033.

26. Kim J, Bartlett S, He L, Nielsen C, Chang A, et al. Morphine-induced receptor endocytosis in a novel knockin mouse reduces tolerance and dependence. Curr Biol, 2008, 18: 129–135.

27. Yu, V. C., S. Eiger, et al. (1990). "Regulation of cyclic AMP by the mu-opioid receptor in human neuroblastoma SH-SY5Y cells." J Neurochem 55(4): 1390-1396.

28. Ballantyne JC. Mao J. Opioid therapy for chronic pain. New England Journal of Medicine. 2003, 349(20):1943-53.

29. Soin A, Cheng J, Brown L, Moufawad S, Mekhail N. Functional outcomes in patients with chronic nonmalignant pain on long term opioid therapy, Pain Practice, 2008, 8:379-384.

30. Chou R, Fanciullo GJ, Fine PG, Adler JA, Ballantyne JC, Davies P, Donovan MI, Fishbain DA, Foley KM, Fudin J, Gilson AM, Kelter A, Mauskop A, O'Connor PG, Passik SD, Pasternak GW, Portenoy RK, Rich BA, Roberts RG, Todd KH, Miaskowski C; American Pain Society-American Academy of Pain Medicine Opioids Guidelines Panel. Clinical guidelines for the use of chronic opioid therapy in chronic noncancer pain. J Pain. 2009, 10(2):113-30.

31. Sharma SK, Klee WA & Nirenberg M, Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. Proc Natl Acad Sci USA. 1975, 72:3092-6.

32. Yu VC, Hochhaus G, Chang FH, Richards ML, Bourne HR & Sadée W., Differentiation of human neuroblastoma cells: marked potentiation of prostaglandin E-stimulated accumulation of cyclic AMP by retinoic acid. J Neurochem 1998, 51:1892-9.

33. T Avidor-Reiss, I Nevo, R Levy, T Pfeuffer, Z Vogel, Chronic opioid treatment induces adenylyl cyclase V superactivation. Involvement of Gbetagamma. J. Biol. Chem., 1996, 271:21309–21315.

34. Alt E, Yan Y, Gehmert S, Song YH, Altman A, Gehmert S, Vykoukal D, Bai X., Fibroblasts share mesenchymal phenotypes with stem cells, but lack their differentiation and colony-forming potential, Biol Cell, 2011, 103(4):197-208.

35. Williams, J.T., Christie, M.J., and Manzoni, O. Cellular and synaptic adaptations mediating opioid dependence. Physiol. Rev. 2001, 81:299–343.

36. Whistler, J.L., Chuang, H.H., Chu, P., Jan, L.Y., and von Zastrow, M. Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron. 1999, 23:737–746.

37. Gholamrezanezhad A, Mirpour S, Bagheri M, Mohamadnejad M, Alimoghaddam K, Abdolahzadeh L, et al. In vivo tracking of 111In-oxine labeled mesenchymal stem cells following infusion in patients with advanced cirrhosis. Nucl Med Biol. 2011;38:961–7.

38. Devine SM, Bartholomew A, Mahmud N, Nelson M, Patil S, Hardy W, et al. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. Exp Hematol. 2001;29:244–55.

39. Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood. 2003;101:2999–3001.

40. Gao J, Dennis JE, Muzic RF, Lundberg M, Caplan AI. The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs. 2001;169:12–20.

41. Karp JM, Leng Teo GS. Mesenchymal stem cell homing: the devil is in the details. Cell Stem Cell. 2009;4:206–16.

42. Leibacher J and Henschler R, Biodistribution, migration and homing of systemically applied mesenchymal stem/stromal cells, Stem Cell Research & Therapy, 2016, PMID:26753925.

43. Yang H, Wang J, Sun J, Liu X, Duan WM, **Qu T*.** A new method to effectively and rapidly generate neurons from SH-SY5Y cells. Neurosci Lett. 2016 Jan 1;610:43-7.

44. Hua Z, Liu L, Shen J, Cheng K, Liu A, Yang J, Wang L, **Qu T,** Yang H, Li Y, Wu H, Narouze J, Yin Y, Cheng J. Mesenchymal Stem Cells Reversed Morphine Tolerance and Opioid-induced Hyperalgesia. Sci Rep. 2016 Aug 24;6:32096. PMID: 27554341

11. Appendices:

- 3 Publications
- 5 Abstracts

UNIVERSITY OF ILLINOIS AT CHICAGO

Department of Psychiatry

Third Annual Research Forum – Extravaganza 2012

POSTER TITLE	Human mesenchymal stem cells attenuate morphine tolerance
DISEASE/KEY WORDS:	hMSCs, morphine, tolerance, cAMP, fluorescent immunoassy
AUTHORS:	Jinhua Sun, Hongna Yang, Tingyu Qu*
MENTEE CATEGORY:	Post-Doctoral Associate **RESEARCH MENTOR:** Tingyu Qu*
BACKGROUND:	Human mesenchymal stem cells (hMSCs) affect the inflammatory milieu and release endogenous analgesic molecules such as met-enkephalins. These findings have led researchers to consider hMSCs as a treatment for various diseased conditions including painful states. Morphine is one of the effective pain killers commonly used in today's clinics. We recently investigated whether hMSCs can attenuate the morphine tolerance in morphine pre-treated SH-SY5Y cells by a cell co-culture system.
METHODS:	SH-SY5Y cell were pre-treated by 10μM morphine for 24h and then co-cultured with different passages of hMSCs or their conditioned media for 36 hours. cAMP level, which is involved in opioid tolerance development as one of the cellular mechanisms, was examined in the tolerated SH-SY5Y cells with and without cell co-cultures by a fluorescent immunoassay.
RESULTS:	As expected, chronic morphine treatment produced an up-regulated level of cAMP in SH-SY5Y cells. hMSCs at early passage (≤passage 5) significantly inhibited the up-regulation of cAMP level in SH-SY5Y cells at the ratio of 1:5 and 1:25 (hMSCs/SH-SY5Y cells), with a gradual decline when the ratio of MSCs/SH-SY5Y cells was further decreased in co-cultures. However, hMSCs at late passage (≥passage 10) and conditioned medium of hMSCs show no significant inhibitory effects on the up-regulation of cAMP level in SH-SY5Y cells, suggesting that the effects of hMSCs in attenuating morphine tolerance seem to be cell-cell contact and cell passage dependent.
CONCLUSIONS:	These results provide new information that hMSCs may attenuate the development of morphine tolerance produced by morphine application in the management of chronic pain.

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Evaluation Of A Non-Invasive System Of Monitoring Minute Ventilation, Tidal Volume,And Respiratory Rate
Freeman, Jenny

Characterization of Pain Behaviors in a Rat Model of Partial Thickness Thermal Injury
Garza, Thomas

Biochemical Correlates for the Analgesic Action Of Curcumin: A Naturally occurring Anti-inflammatory Agent
Greer, Angie

Comparisons of Ibuprofen Dose Responses on Coagulation in Human and Pig Blood
Guerra, Jessica

Dual Mechanism Therapeutics Attenuate Pain Behaviors in a Rat Model of Thermal Injury
Loyd, Dayna

The Relationship Between Ketamine and Posttraumatic Stress Disorder in Burned United States Servicemembers
Petz, Lawrence

Targeted Cell Reprogramming Produces Autologous Analgesic Cells from Human Bone Marrow-derived Mesenchymal Stem Cells
Qu, Tingyu

Hydrocodone Metabolism Following Single Dose Administration
Valtier, Sandra

Analysis of Hydrocodone, Hydromorphone and Norhydrocodone in Plasma using Liquid Chromatography - Tandem Mass Spectrometry (LC/MS/MS)
Valtier, Sandra

Salmon Thrombin Treatment Reduces Protease Activated Receptor 1 Expression following Painful Nerve Root Injury
Winkelstein, Beth

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Targeted cell reprogramming produces autologous analgesic cells from human bone marrow-derived mesenchymal stem cells

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INTRODUCTION

Transplantation of allogeneic adrenal chromaffin tissues, which release endogenous opioid peptides and catecholamines, produces significant analgesic effects in patients with terminal cancer pain. After spinal transplantation, these patients showed increased levels of endogenous opioids and catecholamines in the cerebrospinal fluid with robust and long-lasting pain alleviation accompanied by an inhibition of the development of tolerance to exogenous opioid analgesics. The anti-tolerance effects produced by the transplantation of adrenal chromaffin cells may be attributed to a synergistic action of endogenous analgesic molecules released by the transplanted cells.

Although transplantation of adrenal chromaffin cells demonstrated promise of favorable outcomes for pain relief in patients, there is a very limited availability of suitable human adrenal tissues to serve as grafts. Alternative xenogeneic materials, such as porcine and bovine adrenal chromaffin cells, present problems of immune rejection and possible pathogenic contamination. To overcome these problems, we tested an approach to reprogram human bone marrow-derived mesenchymal stem cells (hMeSCs) using the cellular extracts of porcine chromaffin cells and produced a new type of cells, chromaffin-like cells. These newly-generated chromaffin-like cells acquired some key functional characteristics of adrenal chromaffin cells such as synthesizing and secreting analgesic opioid peptides. Spinal transplantation of these chromaffin-like cells provided significant analgesic effects in an animals. Our ultimate goal is to establish an effective therapy for persistent chronic and intractable pain by utilization of chromaffin-like cells produced from autologous hMeSCs of individual patients. Success of the proposed studies will eventually reduce the need for exogenous opioid medications, with a potential to avoid tolerance as demonstrated in the clinical trials of adrenal chromaffin cell transplantation in humans.

MATERIAL AND METHODS

1)Production of chromaffin-like cells from hMeSCs:

Cell cultures: hMeSCs (Walkersville, MD) were cultured and expanded per the protocol developed in our laboratory (1, 2). Porcine (4 month old) adrenal glands were obtained from a local slaughterhouse and immediately placed in ice-cold Locke's buffer supplemented with antibiotics for processing. The isolation, purification, and culturing for porcine chromaffin cells were performed as described in our previous study (3).
Cell reprogramming: The experimental procedures for cell reprogramming are based on the method developed by Hakelien and Collas (4, 5). Cultured porcine chromaffin cells were washed in PBS and in cold cell lysis buffer (20 mM HEPES, pH 8.2, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors, Sigma-Aldrich), centrifuged at 400g, and resuspended in 1 volume of cell lysis buffer. Cells were then sonicated with a pulse sonicater in short pulses until all cells and nuclei are lysed. The lysate were centrifuged at 15,000g for 15 min at 4°C and the supernatant were aliquoted and stored in liquid nitrogen for later use. Before applying to cell reprogramming, the protein concentration of the cells extracts was determined. To effect cell reprogramming, hMeSCs were reversibly permeabilized with the cholesterol-binding toxin streptolysin O (SLO, Sigma-Aldrich) and $1x10^5$ hMeSCs were incubated in 100µl nuclear and cytoplasmic extracts of $1x10^7$ porcine chromaffin cells containing an ATP-regenerating system (1mM ATP, 10 mM creatine phosphate, 25µg/ml cteatine kinase, 100µM GTP, and 1mM of each NTP) for 1hr at 37°C in a water bath. Following incubation, hMeSCs were resealed with $CaCl_2$ and cultured continuously for the following investigations.
RT-PCR: To characterize the expression of human preproenkephalin (hPPE), a precursor for enkephalin, in reprogrammed and naïve hMeSCs (unreprogrammed), total RNA from cell cultures was isolated at 2 weeks after cell reprogramming with TRIzol reagent (Invitrogen) according to the manufacturer's protocol and treated with RNAase free DNAase (Promega, Madison, WI). The concentration of the RNA was quantified by absorbance at 260 nm. RT-PCR was performed using SuperScript One-Step RT-PCR approach (Invitrogen) using gene specific primer set for hPPE: 5'-ACATCAACTTCCTGGCTTGCGT-3' and 5'-GCTCACTTCTTCCTCATTATCA-3', with β-actin as an internal control. RT-PCR products were quantified using Qgel 1D program (Stratagene) and expressed as hPPE/β-actin ratio. Three independent experiments were performed for this study.
Immunoblot: The production and secretion of Met-enkephalin opioid peptide in cell cultures were examined using an immunoblot assay. In brief, reprogrammed hMeSCs were re-suspended from cell cuture flask and transferred into a 12 well culture plate containing serum-supplemented growth medium at a concentration of $1x10^5$/well, while naïve hMeSCs with the same number of cells ($1x10^5$/well) served as controls. After recovering overnight, the serum-supplemented growth medium for hMeSC cultures was replaced by serum-free medium (DMEM with antibiotic-antimycotic). Twenty-four hours later, medium was collected and purified with YM-30 microcon (Millipore Corp., Bedford, MA). Purified medium samples (200µl for each) obtained from reprogrammed or naïve hMeSCs were applied on Hybond ECL nitrocellurose membrane (Amersham Life Science, Piscataway, NJ) using the Slot Blot Hybridization Manifold (GENEMate, Kaysville, UT). The membranes were incubated with specific Met-enkephalin antibody made in rabbit (1:600, ImmunoStar Inc., Hudson, WI) followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:3000, Amersham Biosciences). The membranes were then revealed with ECL Plus detection reagent and exposed to Hyperfilm ECL (Amersham Biosciences). The films were developed and scanned into a computer. Quantification of immunoblot band density was assessed by densitometric analysis using the NIH image program (ImageJ, NIH). Data was expressed as mean ± SEM of 4 independent experiments.

2) Immunocytochemistry:

Fluorescence immunocytochemical staining was performed on cultured chromaffin-like cells 2 weeks post-cell reprogramming. Naïve hMSCs served as controls. Cell samples in eight-well culture chambers (Thermo Scientific, Swedesboro, NJ) were washed with PBS and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature. Following washing in PBS, cell samples were blocked in PBS buffer containing 0.1% Triton X-100 (Sigma, St Louis, MO) and 3% donkey serum (Jackson ImmunoResearch, West Grove, PA) for 30 min, followed by incubation with mouse anti-TH (1:300; Sigma, St Louis, MO), or rabbit anti-Met-enkephalin (1:600; ImmunoStar Inc., Hudson, WI) antibodies overnight at 4°C. Then, the cells were washed in PBS and incubated with their corresponding secondary antibodies, including FITC-conjugated donkey anti-mouse IgG or rhodamine (TRITC)-conjugated donkey anti-rabbit IgG antibodies for 2 hr at room temperature in the dark. Finally, the cells were washed with PBS, counterstained with DAPI (Vector Labs, Burlingame, CA), and viewed by immunofluorescence microscopy (Zeiss, Jena, Germany).

3) Analgesic effects of chromaffin-like cells in rats:

Adult male Sprague Dawley (SD) rats (n=12, 250–300g) were used for this experiment. SD rats were lightly anesthetized with pentobarbital (35mg/kg, i.p.) and the baseline of foot withdrawal latencies were measured without cell transplantation using high (Aδ nociceptor: 6.5°C/second) and low (C nociceptor: 0.9°C/second) rates of radiant heating on the dorsal surface of the feet. The latencies from the onset of the stimulus to foot withdrawal responses were measured over 1 hour at 10 min intervals. After baseline for foot withdrawal responses was established, $1x10^5$ chromaffin-like cells generated from hMeSCs were transplanted into the rats (n=6) intrathecally under deep anesthesia with pentobarbital (50mg/kg, i.p.). Control rats (n=6) received injection of the same amounts of naïve hMeSCs. Rats were not immunosuppressed. Foot withdrawal latencies were re-measured at 1 week after cell transplantation and then weekly at one week intervals until the latencies returned to baseline. The foot withdrawal latencies were expressed as mean ± SEM. Student's t-test was used to compare the response latencies of chromaffin-like cell transplantation with baseline and that of naïve hMeSC transplantation.

RESULTS

1) Reprogrammed hMeSCs have a significantly enhanced expression for gene hPPE compared to that of naïve hMeSCs (Fig. 1, P<0.01) and a significant augmented release of Met-enkephalin opioid peptide in the serum-free culture medium compared to that of naïve hMeSCs for the same number of the cells (Fig. 2, P<0.01).
2) Immunocytochemical examination demonstrated that these reprogrammed hMeSCs not only expressed Met-enkephalin but also strongly express TH, an enzyme controlling the rate-limiting step of catecholamine biosynthesis, suggesting the potential of these cells to produce catecholamines. A strong immunoreactivity for specific markers of chromaffin cells, Met-enkephalin and TH, was observed in most of the reprogrammed hMeSCs (≥90%, Fig. 3).
3) Transplantation of chromaffin-like cells produced a significant analgesic effects in rats on both Aδ nociceptor- and C nociceptor-evoked responses and the analgesic effects lasted for about 3 weeks without immunosuppression (Fig. 4, P<0.05). Transplantation of naïve hMeSCs in rats showed response latencies similar to those of rats without cell transplantation in previous studies, with no detectable analgesic effects.

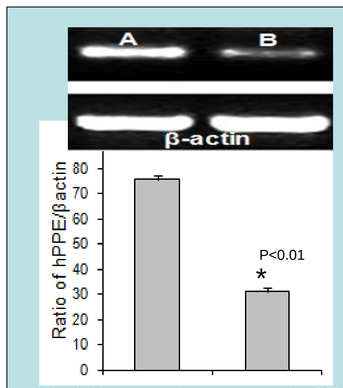

Fig. 1. Upper panel: a representative RT-PCR gel showing that hPPE expression of reprogrammed hMeSCs is significantly up-regulated (lane A) compared with that of unpregrammed hMeSCs (lane B) with β-actin as an internal control. *P<0.01 (Student's t-test).

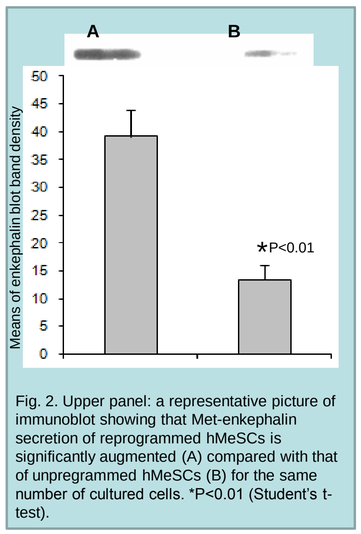

Fig. 2. Upper panel: a representative picture of immunoblot showing that Met-enkephalin secretion of reprogrammed hMeSCs is significantly augmented (A) compared with that of unpregrammed hMeSCs (B) for the same number of cultured cells. *P<0.01 (Student's t-test).

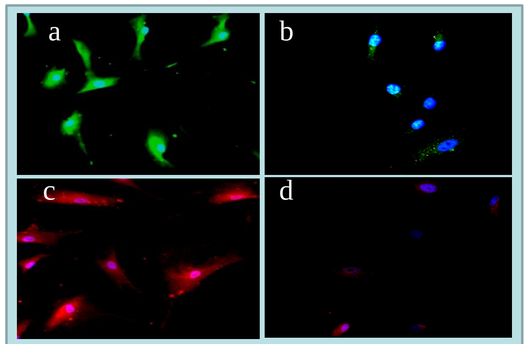

Fig. 3. Micrographs showing that reprogrammed hMeSCs expressed strong immunoreactivity for tyrosine hydroxylase (a, green) and Met-enkephalin (c, red) compared to corresponding unreprogrammed hMeSCs (b & d). DAPI stained the nucleus of all cells (blue).

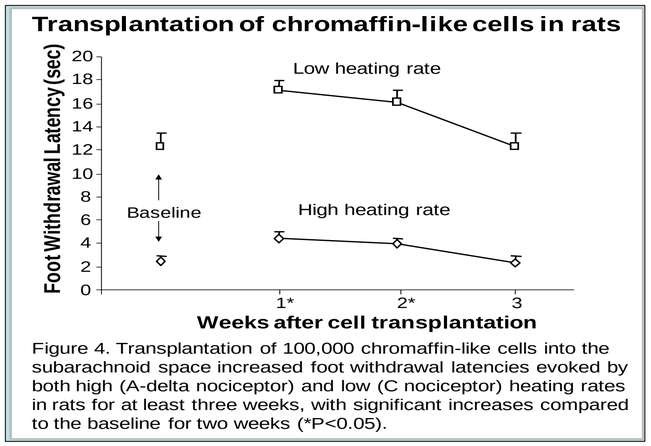

Figure 4. Transplantation of 100,000 chromaffin-like cells into the subarachnoid space increased foot withdrawal latencies evoked by both high (A-delta nociceptor) and low (C nociceptor) heating rates in rats for at least three weeks, with significant increases compared to the baseline for two weeks (*P<0.05).

SUMMARY

Analgesic chromaffin-like cells can be generated from an individual's own tissue-derived stem cells by targeted cell reprogramming and these chromaffin-like cells may serve as potential autografts and provide innovative therapies for chronic pain without immunological problems.

References

1. Qu, T.Y., Dong, X.J., Sugaya, I., Vaghani, A., Pulido, J., Sugaya, K., (2004). Restor. Neurol. Neurosci. 22(6), 459-68.
2. Sugaya I, Qu T, Sugaya K, and Pappas, GD., (2006). Cell Transplant. 15: 225-30
3. Shi, G., Ma, K., Pappas, GD., and Qu, TY., (2008). Neurol. Res., 30(3):217-22.
4. Collas, P., and Hakelien, A. M. (2003). Trends Biotechnol. 21, 354–361
5. Hakelien, A. M., Gaustad, K. G., and Collas, P. (2006). Methods Mol. Biol. 325, 99–114.

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Attenuation of chronic Morphine-mediated cAMP upregulation in SH-SY5Y cells by mesenchymal stem cells

Hongna Yang, Jinhua Sun, Feng Wang, and Tingyu Qu
University of Illinois at Chicago, USA

Abstract

The cAMP upregulation induced by chronic morphine is regarded as one of the molecular mechanisms leading to its tolerance and dependence. In the present work, we differentiated SH-SY5Y cells into neuron-like cells by retinoic acid (RA), pretreated these cells by morphine, the highly addictive drug, and tested their cAMP levels under different conditions, including co-culture with bone marrow-derived human mesenchymal stem cells (hMSCs) and human dermal fibroblast cells (hDFCs). We found that chronic treatment with 10μM morphine led to cAMP upregulation in these differentiated SH-SY5Y cells and the morphine mediated-cAMP upregulation was significantly attenuated by co-culturing with the hMSCs at early passages (P≤5), though this attenuation did not occur in co-cultures with the hMSCs at late passages (P>5) or hDFCs. In addition, hMSCs improved the mu-opioid receptor-mediated endocytosis in SH-SY5Y cells in preventing the development of morphine tolerance. In summary, early passaged hMSCs can successfully inhibit morphine induced cAMP upregulation in RA-differentiated SH-SY5Y cells by cell-cell contact and/or their released molecules, suggesting that hMSCs may serve as valuable therapeutics for treating morphine tolerance and dependence to minimize the risk of drug abuse and addiction. This research is supported by grants of DoD (PR100499P1) & the Boothroyd Foundation to T. QU.

Biography

Hongna Yang is a visiting student of Psychiatry of UIC, from Shandong University in China. Her focus is on the stem cell therapy (NSCs, MSCs) of neurodegenerative diseases and morphine tolerance.

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UNIVERSITY OF ILLINOIS AT CHICAGO

Department of Psychiatry

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POSTER TITLE	A new method to generate neurons effectively from cultured SH-SY5Y cells
DISEASE/KEY WORDS:	Neuronal production, SH-SY5Y cells, neural stem cells, and condition medium
AUTHORS:	Hongna Yang, Yan Li, Feng Wang, and Tingyu Qu
MENTEE CATEGORY:	Post- doc, PhD student, Visiting scientist
RESEARCH MENTOR:	Tingyu Qu

BACKGROUND: It is well known that the differentiated SH-SY5Y cells can be used as a neuronal cell model *in vitro* for neuroscience and neurotoxicity research. Retinoic acid (RA) is the most commonly used inducer for neuronal differentiation of SH-SY5Y cells. However, neuronal production is very low under RA treatment or combination of RA and other chemicals.

METHODS: We explored to use a conditioned medium of human neural stem cells (CM-hNSCs) to culture SH-SY5Y cells in combination with RA for 3 to 7 days to produce neuronal cells.

RESULTS: We found that the neuronal proportion of differentiated SH-SY5Y cells was significantly iicreased after 3 and 7 day treatment of CM-hNSCs and RA compared to that with only RA treatment, in which about 90% of differentiated cells showing positive beta-III tubulin staining, a well-accepted neuronal marker. In addition, maturation of the neurons differentiated from SH-SY5Y cells following CM-hNSCs and RA treatment was greatly improved, with long and multiple cell process as well as fiber development.

CONCLUSIONS: It is the first study to use CM-hNSCs for promoting neuronal differentiation of RA treated SH-SY5Y cells. Our new method can effectively induce neuronal differentiation of SH-SY5Y cells and increase the proportion of neurons in these differentiated cells.

This research is supported by grants of DoD (PR100499P1) & the Boothroyd Foundation to T. QU.

UNIVERSITY OF ILLINOIS AT CHICAGO

Department of Psychiatry

Fifth Annual Research Forum – Extravaganza 2014

POSTER TITLE	Mesenchymal stem cells inhibit morphine-mediated tolerance development
DISEASE/KEY WORDS:	Stem cells; morphine-mediated tolerance; enkephalin; pain therapy
AUTHORS:	Yan Li, Feng Wang, HongNa Yang, and Tingyu Qu
MENTEE CATEGORY:	Visiting scientist, Post- doc, PhD student
RESEARCH MENTOR:	Tingyu Qu

BACKGROUND: Previous studies demonstrated that transplantation of adrenal chromaffin cells in humans play an important role in the analgesia and the inhibition of opioid tolerance, which may be attributed to a synergistic action of endogenous molecules such as opioid peptides and catecholamines released by these cells. It has been reported recently that mesenchymal stem cells (MSCs) express preproenkephalin (PPE), a protein precursor of the opioids peptides Met- and Leu-enkephalins, and are also capable of spontaneously releasing Met-enkephalin and anti-inflammatory cytokines in cultured condition, these cells may also produce analgesic and anti-tolerance effects to chronic opioids.

METHODS: We investigated the potential effect of MSCs to the development of morphine-induced tolerance *in vitro* by a co-culture system of human MSCs (hMSCs) and the neuronally-differentiated SH-SY5Y cells and *in vivo* by spinal transplantation of rat MSCs (rMSCs) in a rat pain model. We also investigated the expression level of enkephalins in the culture supernatant of hMSCs by ELISA and in situ immunofluorescence.

RESULTS: We found that chronic treatment with 10μM morphine led to cAMP upregulation in neuronally-differentiated SH-SY5Y cells, a well-accepted cell response for morphine tolerance and that naïve hMSCs at the early passage ($P<5$) demonstrated an inhibition to the development of morphine tolerance and resulted in significant reduction to the morphine-induced cAMP production in these neuronal cells differentiated from SH-SY5Y cells in our co-culture system. However, this inhibition did not occur in co-cultures of neuronally-differentiated SH-SY5Y cells with the hMSCs at late passage ($P>5$). The level of enkephalins in both of the supernatant of hMSC culture and in situ was significantly augmented compared to the controls. More importantly, spinal transplantation of rMSCs at ealy stage ($P<3$) produced significant analgesic and robust anti-tolerance effects in the model rats with pain.

CONCLUSIONS: Our studies, both in vitro and in vivo, reveled that MSCs may serve as valuable therapeutic cells for treating morphine tolerance and dependence to minimize the risk of drug abuse and addiction.

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Targeted Cell Reprogramming Produces Analgesic Chromaffin-Like Cells From Human Mesenchymal Stem Cells

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Transplantation of allogeneic adrenal chromaffin cells demonstrated the promise of favorable outcomes for pain relief in patients. However, there is a very limited availability of suitable human adrenal gland tissues, genetically well-matched donors in particular, to serve as grafts. Xenogeneic materials, such as porcine and bovine adrenal chromaffin cells, present problems; for instance, immune rejection and possible pathogenic contamination are potential issues. To overcome these challenges, we have tested the novel approach of cell reprogramming to reprogram human bone marrow (BM)-derived mesenchymal stem cells (hMSCs) using cellular extracts of porcine chromaffin cells. We produced a new type of cell, chromaffin-like cells, generated from the reprogrammed hMSCs, which displayed a significant increase in expression of human preproenkephalin (hPPE), a precursor for enkephalin opioid peptides, compared to the inherent expression of hPPE in naive hMSCs. The resultant chromaffin-like cells not only expressed the key molecular markers of adrenal chromaffin cells, such as tyrosine hydroxylase (TH) and methionine enkephalin (Met-enkephalin), but also secreted opioid peptide Met-enkephalin in culture. In addition, intrathecal injection of chromaffin-like cells in rats produced significant analgesic effects without using immunosuppressants. These results suggest that analgesic chromaffin-like cells can be produced from an individual's own tissue-derived stem cells by targeted cell reprogramming and also that these chromaffin-like cells may serve as potential autografts for chronic pain management.

Key words: Mesenchymal stem cells; Adrenal chromaffin cells; Cell reprogramming; Pain management; Autologous stem cells

INTRODUCTION

Transplantation of allogeneic adrenal chromaffin cells provides significant pain relief in patients with intractable cancer pain and in patients experiencing allodynia, a hallmark of neuropathic pain (1,11,13,25). There is often long-term pain relief without analgesic tolerance (1 year in humans) (24). Following adrenal chromaffin cell transplantation, application of exogenous opiates further alleviate pain without dosage escalation (25), that is, there was a stabilization of exogenous analgesic intake in these patients, strongly indicating that chromaffin cell grafts ameliorated the problem of opioid tolerance (12,13). These adrenal chromaffin cells release a "cocktail" of endogenous analgesic substances, including enkephalins, catecholamines, γ-amino butyric acid, indolalkylamines, and other neuropeptides (15,42). The analgesic effects of chromaffin cells can be partially reversed by intrathecal injection of either the opioid antagonist naloxone or the adrenergic antagonist phentolamine (9,32,33), suggesting that these analgesic effects are mediated by the opioids and catecholamines released by these cells. The antitolerance effects produced by the transplantation of adrenal chromaffin cells may be attributed to the synergistic action of the endogenous molecules released by these transplanted cells.

Although transplantation of allogeneic chromaffin cells demonstrates a viable modality of effective treatment for relieving pain and suffering in patients with the promise of ameliorating opioid tolerance, clinical practice has been hindered due to the limited availability of suitable human adrenal gland tissues. Mature chromaffin cells are postmitotic when they produce enkephalins and catecholamines. Expansion of these cells in culture is not possible. Xenogeneic materials, such as bovine and porcine chromaffin cells, have been extensively studied as potential alternative materials to human chromaffin cells. Transplantation of these xenogeneic cells into the spinal subarachnoid space produces antinociceptive effects on both Aδ and C fiber-mediated responses in rodents and in nonhuman primates (16,23,32–34,40) with a gradual

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decline in analgesic efficacy that can be prolonged by administration of immunosuppressants (23). Although chromaffin cells themselves are not very immunogeneic and highly purified chromaffin cells may minimize immunorejection (4,20), these experimental results suggest that, in transplants, xenogeneic chromaffin cells elicit immunological host rejection and that immunosuppressive therapy is necessary for enhancing long-term graft survival to extend the analgesic effect of these transplants. In addition, there has been concern regarding pathogen contamination by these xenogeneic materials, such as bovine spongiform encephalopathy in bovine chromaffin cell transplantation. Thus, the use of xenogeneic chromaffin cells presents serious problems.

The emerging approach of cell extract-based cell reprogramming developed by Håkelien and Collas et al. (3,5,8) represents a novel technology to furnish the phenotypic characteristics of the target cells to the reprogrammed cells for therapeutic purposes. We previously (41) reported that bone marrow (BM)-derived human mesenchymal stem cells (hMSCs) hold the inherent gene expression of preproenkephalin (PPE), a precursor for enkephalin opioid peptides, such as methionine- (Met-) and leucine- (Leu-) enkephalins, and in culture are able to release a low basal level of Met-enkephalin, a major neurotransmitter that plays an important role in analgesia by activating opioid receptors (15,33). To address the clinical needs for new and safe alternatives to adrenal chromaffin cells, recently, we further reprogrammed these hMSCs with nuclear and cytoplasmic extracts of porcine chromaffin cells and examined the phenotypic development and functional changes of the reprogrammed cells. RT-PCR assays revealed that the expression of human PPE (hPPE) in the reprogrammed hMSC population was greatly enhanced compared to that of naive hMSCs (unprogrammed). Immunoblot analysis confirmed that secretion of Met-enkephalin in serum-free culture of reprogrammed hMSCs was significantly augmented compared to secretions released by naive hMSCs for the same number of cells under identical conditions. Immunocytochemical examination demonstrated strong immunoreactivity in the reprogrammed hMSCs for Met-enkephalin and tyrosine hydroxylase (TH), specific markers for adrenal chromaffin cells. By targeted cell reprogramming, we have successfully developed a new type of cells from the reprogrammed hMSCs, which demonstrates the key phenotypic and functional features of adrenal chromaffin cells and thus termed these cells "chromaffin-like cells" (37,38). Transplantation of these chromaffin-like cells into the spinal intrathecal space of animals produced significant analgesic effects on both Aδ nociceptor- and C nociceptor-mediated responses in a rat thermal pain model. The analgesic effects lasted for 3 weeks without immunosuppression. Preliminary data from some of this work have been reported previously in abstracts (37,38).

MATERIALS AND METHODS

Chromaffin Cell Isolation, Purification, and Culture

Sixteen male and female porcine (1 year old) adrenal glands were obtained from a local slaughterhouse and immediately placed in ice-cold Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 5 mM HEPES, pH 7.4; all components are from Sigma, St. Louis, MO, USA) supplemented with 100 UI/ml penicillin, 100 mg/ml streptomycin, and 0.125 ml/ml fungizone (PSF; Sigma-Aldrich) for transport. Preparation of chromaffin cells was carried out using a method reported by us previously (39) with modifications. In brief, adrenal glands were manually perfused three times with warmed (37°C) Locke's buffer followed by digestion of connective tissue with 0.125% collagenase A (Boehringer Mannheim, Mannheim, Germany) in Locke's buffer for 3×5 min at 37°C. At the end of the digestion period, the chromaffin cells were isolated from the dissected medulla by mechanical dissociation. The harvested cells were filtered through a 70-μm nylon mesh (BD Falcon, Bedford, MA, USA) and then centrifuged at 150×*g* for 10 min in Locke's buffer. The resulting cells were purified on 39.47% Percoll gradients (Pharmacia Biotech, Uppsala, Sweden) by centrifugation at 22,500×*g* for 20 min. The portion of the gradients containing purified chromaffin cells was harvested by aspiration and washed three times by centrifugation at 150×*g* in Locke's buffer. Freshly isolated chromaffin cells were suspended and plated in 75-cm^2 culture flasks (Corning, Cambridge, MA, USA) containing Dulbecco's modified Eagle's medium/F-12 (DMEM/F12, 1:1; Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma) and antibiotics (PSF). These chromaffin cells were maintained at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA, USA) fed by replacing culture media twice per week and used for experiments within 4 weeks after culturing because mature chromaffin cells are postmitotic and do not survive longer in cultured conditions.

hMSC Culture

In total, four samples of hMSCs (three males, ages 22, 25, and 32; one female, age 50), which are negative for surface markers associated with hematopoietic cells [e.g., cluster of differentiation 11b (CD11b), CD33, CD34 and CD133 antigens], were obtained from Cambrex (Walkersville, MD, USA). hMSCs were cultured and expanded using the protocol previously developed in our laboratory (30,39,41). In brief, hMSCs were plated in 75-cm^2 culture flasks at a concentration of 1×10^5 cells/cm^2 and cultured in 20 ml of serum-supplemented growth medium consisting of DMEM, an antibiotic–antimycotic mixture (1:100, Invitrogen, Carlsbad, CA, USA), and FBS (Stem Cell Technologies, Vancouver, BC, Canada), incubated at

37°C in a 5% CO_2 humidified incubation chamber, and fed by replacing half of the culture media twice a week. Cells were passaged by incubating with 0.05% trypsin–EDTA (Gibco) for 5 min at room temperature to gently release the cells from the surface of the culture flask after reaching about 80% confluency. Culture medium was added to stop trypsinization, and the cells were centrifuged at $350 \times g$ for 5 min at room temperature, resuspended, and transferred into new culture flasks at a concentration of 1×10^5 cells/cm^2 for continuous culture and expansion to reach a sufficient number of cells. hMSCs that underwent less than 10 passages were used for the experiments.

Reprogramming hMSCs With the Extracts of Porcine Chromaffin Cells

The technology for cellular extract-based cell reprogramming, originally reported by Håkelien and Collas et al. (3,5,8), was applied by us with some modifications (37,38).

Preparation of Porcine Chromaffin Cell Extracts. Cultured porcine chromaffin cells were counted and washed twice in PBS (Sigma) and in cell lysis buffer (20 mM HEPES, pH 8.2, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors; all from Sigma), sedimented at $400 \times g$, resuspended in 1 volume of cell lysis buffer, and incubated for 30 min on ice. Cell samples were then sonicated in 200 µl of aliquot on ice with a pulse sonicator (PowerGen 125, Fisher Scientific) in short pulses until all cells and nuclei were lysed and confirmed by microscopic observation. The lysate was centrifuged at $15{,}000 \times g$ for 15 min at 4°C. The supernatant was aliquoted and stored in liquid nitrogen for later use. Before applying cell reprogramming, the protein concentrations of the cell extracts were determined (~30 mg/ml).

Permeabilization of hMSCs With SLO. hMSCs were resuspended from the culture, washed in Ca^{2+}- and Mg^{2+}-free PBS (Mediatech, Inc., Manassas, VA, USA), and centrifuged at $120 \times g$ for 5 min at 4°C. The collected hMSCs were resuspended in aliquots of 1×10^5 hMSCs/100 µl of Ca^{2+}- and Mg^{2+}-free PBS in 1.5-ml tubes. Cell samples were permeabilized with streptolysin O (SLO; Sigma-Aldrich) at a final concentration of 200 ng/ml and incubated in an H_2O bath at 37°C for 50 min with occasional agitation to mix the cells. The cell samples were then placed on ice, diluted with 200 µl of cold PBS, and sedimented at $150 \times g$ for 5 min at 4°C.

Reprogramming hMSC With the Extracts of Porcine Chromaffin Cells. The reversibly permeabilized hMSCs (1×10^5) were resuspended in 100 µl of the extracts of porcine chromaffin cells in a 1.5-ml tube containing an ATP-regenerating system (1 mM ATP, 100 µM GTP, and 1 mM of each NTP, 10 mM creatine phosphate, 25 µg/ml creatine kinase) (Sigma, St. Louis, MO, USA). Cell samples were incubated in an H_2O bath at 37°C for 1 h with occasional agitation. To reseal the membranes of hMSCs, 1 ml of DMEM containing 2 mM of $CaCl_2$ (Sigma) and antibiotics were added to the tube and incubated at 37°C for an additional 1 h. Finally, $CaCl_2$-containing DMEM was replaced by fresh DMEM with 10% FBS, and reprogrammed hMSCs were transferred to a 24-well plate (BD, Franklin Lakes, NJ, USA) at a concentration of 1×10^4 cells per well or into a 75-cm^2 culture flask at a concentration of 1×10^5 cells/cm^2 and incubated at 37°C in a 5% CO_2 humidified incubation chamber (Fisher). Twenty-four hours later, the floating cells were removed. The reprogrammed hMSCs grew well in culture and were expanded continuously, fed by replacing half of the culture media twice per week, and underwent regular cell passages when confluence was reached. Cells between passage 2 (1 week after cell reprogramming) and passage 4 (2 weeks after cell reprogramming) were used for experiments, and the viability of chromaffin-like cells was examined by trypan blue (Lonza, Walkersville, MD, USA), exclusion to be ≥95% at the time point before each experiment.

RT-PCR

The expression of hPPE genes in the population of chromaffin-like cells was analyzed by RT-PCR 1 week (passage 2) after cell reprogramming. Naive hMSCs with the same number of passages served as controls. RNAs from the cells were isolated using TRIzol reagents (Invitrogen) according to the manufacturer's protocol and treated with RNAase-free DNAase (Promega, Madison, WI, USA). The concentration of RNA was quantified by absorbance at 260 nm. RT-PCR was performed using a SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen) by means of specific primer sets for gene hPPE (forward: 5′-AC ATCAACTTCCTGGCTTGCGT-3′ and reverse: 5′-GCT CACTTCTTCCTCATTATCA-3′) and β-actin (forward: 5′-GACAGGATGCAGAAGGAGAT-3′ and reverse: 5′-TT GCTGATCCACATCTGCTG-3′). RT-PCR products were quantified using the Qgel 1D program (Stratagene, Cambridge, UK) and expressed as hPPE/β-actin (an internal control) ratio. In total, three independent experiments were performed for this experiment. Statistical analysis was performed using Student's *t* tests at a significance of $p < 0.05$.

Immunoblot

The production and secretion of Met-enkephalin opioid peptides in chromaffin-like cell culture was examined at 1 week (passage 2) following cell reprogramming using an established protocol of immunoblot assay as we performed previously (41). Cultured hMSCs with the same number of passages served as controls. In brief, cultured cells were resuspended from cell culture flasks and transferred onto a 12-well culture plate (BD) containing

serum-supplemented growth medium at a concentration of 1×10^5 cells/well. After recovering overnight, the serum-supplemented culture medium was replaced by serum-free culture medium. Twenty-four hours later, the medium was collected and purified with YM-30 microcon (Millipore Corp., Bedford, MA, USA). Purified medium samples (200 µl for each) obtained from chromaffin-like cells and naive hMSCs were applied to Hybond ECL nitrocellulose membranes (Amersham Life Science, Piscataway, NJ, USA) using the Slot Blot Hybridization Manifold (GENEMate, Kaysville, UT, USA). The membranes were blocked with 3% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) in PBS containing 0.5% Tween 20 (PBST; Sigma) for 2 h at room temperature and then incubated with a specific Met-enkephalin rabbit antibody (1:600, ImmunoStar Inc., Hudson, WI, USA) overnight at 4°C. After washing three times with PBST, the membranes were incubated with anti-rabbit IgG peroxidase-linked species-specific whole donkey antibody (1:3,000, Amersham Biosciences, Piscataway, NJ, USA) for 90 min at room temperature. The membranes were washed with PBS, incubated with an ECL Plus detection reagent for 5 min, and then exposed to Hyperfilm ECL (Amersham Biosciences). The films were developed and scanned by a computer. The quantification of immunoblot band density was assessed by densitometric analysis using the NIH image program (ImageJ, NIH, Bethesda, MD, USA). Data were expressed as the mean ± SEM of four independent experiments. Student's *t* tests were performed to compare the levels of Met-enkephalin released by chromaffin-like cells with the levels released by naive hMSCs for the same number of the cells under the same condition of the serum-free cultures. Significance was set at $p < 0.05$.

Immunocytochemistry

Fluorescence immunocytochemical staining was performed on cultured chromaffin-like cells 2 weeks (passage 4) post-cell reprogramming. Naive hMSCs served as controls. Cell samples in eight-well culture chambers (Thermo Scientific, Swedesboro, NJ, USA) were washed with PBS and fixed in 4% paraformaldehyde (Sigma) in PBS (pH 7.4) for 20 min at room temperature. To examine the proliferative potential of chromaffin-like cells, cell samples were incubated with 1 µM of bromodeoxyuridine (BrdU; Sigma) for 24 h and then treated with 2 N HCL (Fisher Scientific, Fair Lawn, NJ, USA) for 30 min. Following washing in PBS, cell samples were blocked in PBS buffer containing 0.1% Triton X-100 (Sigma-Aldrich) and 3% donkey serum (Jackson ImmunoResearch) for 30 min, followed by incubation with sheep anti-BrdU (1:500; Fitzgerald, Concord, MA), mouse anti-TH (1:300; Sigma-Aldrich), or rabbit anti-Met-enkephalin (1:600; ImmunoStar Inc., Hudson, WI, USA) antibodies overnight at 4°C. Then the cells were washed in PBS and incubated with their corresponding secondary antibodies, including rhodamine (TRITC)-conjugated donkey anti-sheep IgG (1:200; Jackson ImmunoResearch), fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG, and rhodamine (TRITC)-conjugated donkey anti-rabbit IgG antibodies for 2 h at room temperature in the dark. Finally, the cells were washed with PBS, counterstained with DAPI (Vector Labs, Burlingame, CA, USA), and viewed by immunofluorescence microscopy (Zeiss, Jena, Germany). The number of cells fluorescently immunostained by a specific antibody and the number of cell nuclei stained by DAPI were counted in five randomly selected microscopic fields at 200× magnification. In total, three independent experiments were performed, and about 50–100 cells were counted for each experiment. The ratio of the cells fluorescently immunostained by specific antibodies to the total number of counted cells was recorded.

Pain Behavioral Tests

All animal experiments were performed in accordance with federal guidelines for proper animal care and an approved Institutional Animal Care and Use Committee protocol. In total, 12 adult male Sprague–Dawley (SD) rats (250–300 g body weight; Charles River Laboratories, Hollister, CA, USA) were used. Foot withdrawal latencies were measured before cell transplantation using high (Aδ nociceptor: 6.5°C/s) and low (C nociceptor: 0.9°C/s) rates of radiant heating on the dorsal surface of the feet according to the methods previously reported by us (16,17). The latencies from the onset of the stimulus to foot withdrawal responses were measured over 1 h at 10-min intervals. Chromaffin-like cells cultured 2 weeks (passage 4) after cell reprogramming were used. After the baseline for foot withdrawal responses was established, a single dose (1×10^5) of chromaffin-like cells in 20 µl of DMEM was injected intrathecally between the L4 and L5 lumbar vertebrae levels into the rats ($n = 6$) under anesthesia with pentobarbital (50 mg/kg, IP; Abbott Laboratories, North Chicago, IL, USA). We have observed previously that transplantation of this amount of chromaffin-like cells is appropriate to produce optimal analgesic responses in rats (37). Control rats ($n = 6$) received injections of the same amounts of naive hMSCs. Immunosuppressants were not used for cell transplantation. Foot withdrawal latencies were remeasured 1 week following cell transplantation and then weekly at 1-week intervals until latencies returned to baseline. Foot withdrawal latencies were expressed as the mean ± SEM. Data from baseline (before cell transplantation) and response latencies at different time points after cell transplantation were compared and analyzed using *t* tests followed by Bonferroni post hoc correction. Significance was set at $p < 0.05$.

RESULTS

hPPE Gene Expression of Chromaffin-Like Cells

One week after cell reprogramming with the cellular extracts of porcine chromaffin cells, RT-PCR was performed to examine the expression of hPPE. The molecular size of the RT-PCR product for the hPPE gene fragment was 425 bp. As expected, naive hMSCs demonstrated a low level of inherent hPPE gene expression. The chromaffin-like cells generated from the reprogrammed hMSCs showed a significantly enhanced expression profile for the gene hPPE compared to that of naive hMSCs ($p > 0.01$) (Fig. 1), suggesting that cell reprogramming further increases the expression of hPPE genes in the population of reprogrammed hMSCs.

Met-Enkephalin Secretion of Chromaffin-Like Cells

In parallel to the time point examined for the expression of hPPE genes by RT-PCR (1 week post-cell reprogramming), the serum-supplemented culture medium for chromaffin-like cell and naive hMSC cultures was replaced by a serum-free culture medium. Twenty-four hours later, the medium was collected and purified for Met-enkephalin detection by immunoblot assays. As shown in Figure 2, the level of Met-enkephalin released

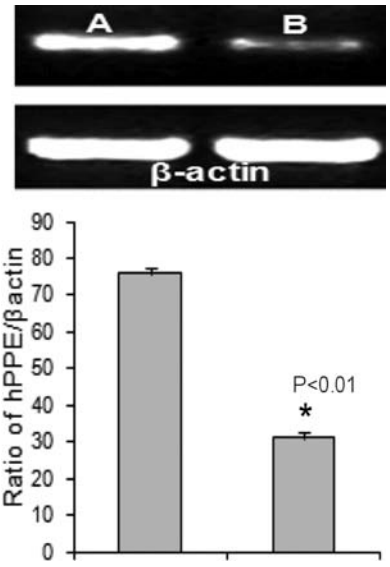

Figure 1. The expression of hPPE gene in the chromaffin-like cells was analyzed by RT-PCR at 1 week (passage 2) after cell reprogramming. Naive human mesenchymal stem cells (hMSCs) with the same number of passages served as controls. RT-PCR products (425 bp) were quantified using the Qgel 1D program (Stratagene, Cambridge, UK) and expressed as human preproenkephalin (hPPE)/β-actin (an internal control) ratio. In total, three independent experiments were performed. Statistical analysis showed that hPPE expression of chromaffin-like cells (A) is significantly upregulated compared to that of naive hMSCs (B) (*$p < 0.01$, Student's *t* test).

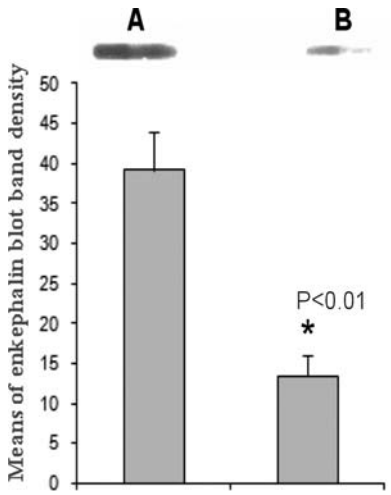

Figure 2. The secretion of Met-enkephalin opioid peptides of chromaffin-like cells (1×10^5 cells) in serum-free culture was examined by an immunoblot at 1 week (passage 2) after cell reprogramming. Naive hMSCs (1×10^5 cells) with the same number of passages served as controls. Twenty-four hours after culturing, the medium sample was collected and purified for immunoblot assay. The quantification of immunoblot band density was assessed by densitometric analysis using the NIH image program (ImageJ). Data were expressed as the mean ± SEM of four independent experiments. Statistical analysis showed that the Met-enkephalin secretion of chromaffin-like cells (A) is significantly augmented compared to that of naive hMSCs (B) for the same number of cells under the same culture condition (*$p < 0.01$, Student's *t* test).

by chromaffin-like cells was significantly augmented compared to that released by naive hMSCs for the same number of cells (1×10^5 cells/well) in serum-free cultures ($p < 0.01$). Although naive hMSCs were able to produce and release a low level of Met-enkephalin into the serum-free medium, augmented production and secretion of Met-enkephalin opioid peptides in chromaffin-like cells were consistently observed in each of the four independent experiments.

Immunocytochemical Examination of Chromaffin-Like Cells

Following cell reprogramming, morphological changes in hMSCs were observed within the first few days, that is, reprogrammed hMSCs became smaller and rounder. Five days later, the cells reverted to fibroblast-like shapes. These cells expanded in culture at a speed slightly slower (doubling time: about 84 h) in the first week and recovered to a normal dividing rate (doubling time: about 72 h) similar to that of naive hMSCs. Two weeks after cell reprogramming, immunocytochemical examination showed that most of the resultant chromaffin-like cells (≥90%) expressed a strong immunoreactivity for Met-enkephalin and TH (Fig. 3), specific cytoplasmic markers for adrenal

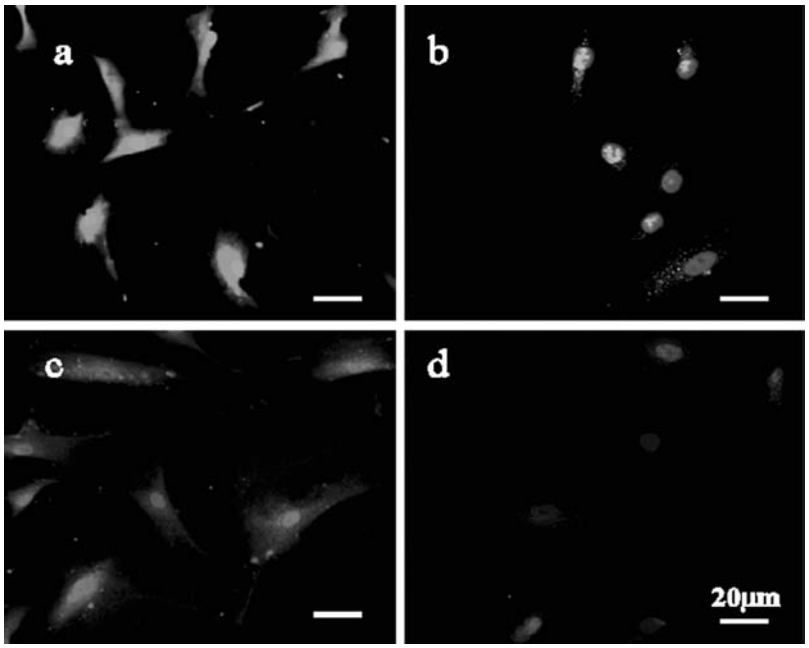

Figure 3. Fluorescence immunocytochemical staining was performed on cultured chromaffin-like cells 2 weeks post-cell reprogramming (passage 4). Naive hMSCs with the same number of passages served as controls. Micrographs showed that chromaffin-like cells expressed strong immunoreactivity for tyrosine hydroxylase (a) and Met-enkephalin (c), while naive hMSCs expressed weak immunoreactivity for tyrosine hydroxylase (b) and Met-enkephalin (d). DAPI stained the nucleus of all cells. Consistent results were received in three independent experiments. Scale bar: 20 μm.

chromaffin cells. Interestingly, BrdU-positive staining was detected in a subpopulation of chromaffin-like cells (data not shown), suggesting that some chromaffin-like cells may have retained a similar proliferative capability as that of hMSCs and could be expandable in cell cultures. These chromaffin-like cells remained stable phenotypes and expanded in cultures with an average cell doubling time of about 72 h up to 1 month, undergoing about eight passages. We did not maintain the culture of the cells past the 1-month time point after cell reprogramming.

Analgesic Effects of Chromaffin-Like Cells

The analgesic effects of the chromaffin-like cells to a noxious thermal stimulus were investigated with the Aδ and C fiber-mediated foot withdrawal latency tests in rats. Transplantation of chromaffin-like cells into the subarachnoid space of rats produced remarkable analgesic effects on both Aδ and C fiber-mediated responses, which were evoked by a high and a low heating rate, respectively, and increased foot withdrawal latencies for 3 weeks in the absence of immunosuppression, with significant increases compared to the baselines of foot withdrawal latencies for 2 weeks (Fig. 4) ($p<0.01$). The analgesic effects of chromaffin-like cells were greater for C fiber- than for Aδ fiber-mediated responses. The maximum analgesic effects were observed in the first week following cell transplantation, and analgesic efficacy declined gradually with time. Control rats with naive hMSC transplantation showed similar response latencies to those measured previously in rats without cell transplantation (data not shown). No adverse effect was observed in the rats either with chromaffin-like cell or with naive hMSC transplantation.

DISCUSSION

Cellular extract-based cell reprogramming technology makes it possible to develop new types of cells by reprogramming one type of cells with extracts derived from a chosen targeted cell type to furnish reprogrammed cells with the phenotypic characteristics of the target cells (3,5,8). For example, incubation of a cell line, 293T fibroblasts, in the nuclear and cytoplasmic extracts of human T-cells resulted in the reprogrammed fibroblast cells taking on T-cell properties, expressing T-cell-specific surface molecules, and assembling the interleukin-2 receptor in response to T-cell receptor CD3 stimulation—a complex regulatory function (8). By using a similar approach, a related study demonstrated the induction of the pancreas-specific genes pancreatic and duodenal homeobox 1 (Pdx1) and insulin in rat primary fibroblasts treated with an extract of rat insulinoma cells (6). In the current studies, we reprogrammed hMSCs with nuclear and cytoplasmic extracts from porcine chromaffin cells, and the reprogrammed hMSCs demonstrated

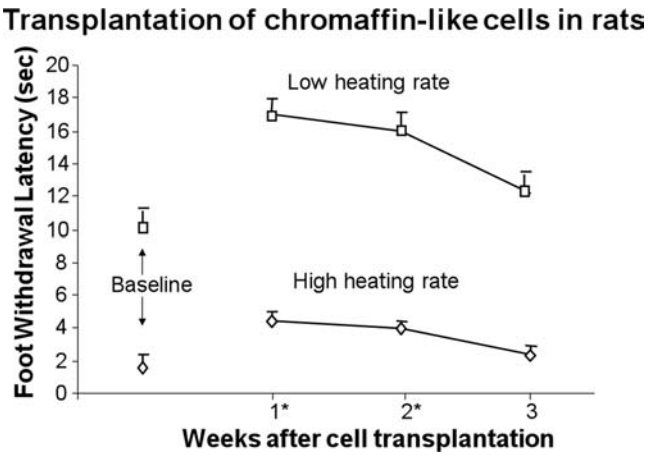

Figure 4. Effects of intrathecal transplantation of a single dose (1×10^5) of chromaffin-like cells on foot withdrawal latencies evoked by low (C nociceptor: 0.9°C/s, squares) and high (Aδ nociceptor: 6.5°C/s, diamonds) heating rates on the dorsal surface of the feet in rats ($n=6$). Foot withdrawal latencies were measured before cell transplantation (baseline) and remeasured 1 week and then weekly at 1-week intervals following cell transplantation. Data from baseline and response latencies at different time points after cell transplantation were expressed as the mean ± SEM and compared using *t* tests. Follow-up analysis was performed by Bonferroni post hoc tests. Results showed that transplantation of chromaffin-like cells increased foot withdrawal latencies evoked by both high and low heating rates for at least 3 weeks, with significant increases compared to baseline for 2 weeks (*$p<0.01$, *t* tests and Bonferroni correction).

phenotypic and functional characteristics similar to those of true chromaffin cells.

As previous studies have shown, hMSCs are a subset of self-renewing multipotent stem cells and are capable of differentiating into various mesenchymal cell lineages, including bone, cartilage, fat, tendon, and other connective tissues (18,26,28). Several studies have reported that hMSCs can also transdifferentiate into a diverse family of cell types unrelated to their phenotypic embryonic origin, including muscle and hepatocytes (14,19,27), as well as neural cells (2,30,35). Recent studies, including ours, have revealed that hMSCs display an inherent gene expression of hPPE and spontaneously release a low level of Met-enkephalin in culture (29,41). These cells are preferred candidates for our targeted cell reprogramming not only because of their latent capability to produce analgesic substances and plasticity for multitransdifferentiation but also because they are relatively easy to isolate from an individual's own tissues and are able to be expanded in culture with a regular doubling time as well as low levels of senescence during repeated passages. We choose porcine adrenal chromaffin cells as reprogramming materials because these cells share characteristics with human adrenal chromaffin cells in many respects, such as synthesizing and releasing opioid peptides and other pain-inhibitory compounds, including enkephalins and catecholamines (16,40). Also, compared with bovine chromaffin cells, porcine chromaffin cells are more potent in producing analgesia as transplants (16). In addition, porcine chromaffin cells are considered a safe source of cells that are readily available in large quantities—in fact, pigs have been cloned and are now being bred for whole-organ transplantation in humans (21,22).

Consistent with previous studies (29,41), our results demonstrate that naive hMSCs are able to express a low level of hPPE genes and to release a basal level of Met-enkephalins into a serum-free culture medium. However, chromaffin cell extract-based cell reprogramming significantly increases the expression of hPPE and the production and release of analgesic molecule Met-enkephalin, a neurotransmitter that plays a major role in analgesia by activating peripheral opioid receptors, in reprogrammed hMSCs ($p<0.01$) (Figs. 1 and 2). As shown by our immunocytochemical examination, about 90% of the resultant chromaffin-like cells expressed strong Met-enkephalin and TH (Fig. 3), key markers of chromaffin cells. We did not examine the levels of catecholamines released by chromaffin-like cells in this study. However, TH is an enzyme controlling the rate-limiting step of catecholamine biosynthesis and is specifically found in the cytoplasmic matrix of cells containing catecholamines (31,36); positive immunoreactivity for TH may suggest that these chromaffin-like cells have the potential to produce catecholamines. More interestingly, BrdU immunoreactivity was detected in a subpopulation of chromaffin-like cells (30%), suggesting that these cells may have retained the proliferative properties of hMSCs and may make these cells even more valuable because generation of dividing

cells that are expandable in culture would provide a sufficient quantity of such cells for targeted use. In practice, the analgesic potential and secretion activity of chromaffin-like cells for enkephalins can be further manipulated in continuous cultures and thus become more powerful by using targeted gene transfection and/or cell fusion techniques, as demonstrated in our previous studies (39,41). As revealed by RT-PCR, immunocytochemistry, and immunoblot analyses in our studies, reprogrammed hMSCs demonstrated similar phenotypic and functional characteristics of chromaffin cells by targeted cell reprogramming. Although the mechanism for cell reprogramming is not fully understood, it is suggested that nuclear and cytoplasmic extracts may contain regulatory components that mediate alterations in the gene expression profile of the target genome and promote the nuclear importation of nuclear regulatory components (7,10). It is likely that chromaffin cell extract-based cell reprogramming switches hMSCs from a mesenchymal program to a chromaffin-like program.

We further investigated the analgesic effects of chromaffin-like cells in vivo by spinal transplantation of the cells into the subarachnoid space of the rats. As shown in Figure 4, transplantation of chromaffin-like cells produced remarkable analgesic effects and significantly increased the foot withdrawal latencies mediated by both high (Aδ nociceptor) and low (C nociceptor) heating rates ($p<0.01$) (Fig. 4). The analgesic effects of chromaffin-like cells lasted for 3 weeks in rats without immunosuppression. Although we did not make observations on the secretion of chromaffin-like cells past the 1-week time point of cell reprogramming in our in vitro studies, the analgesic effects of chromaffin-like cells demonstrated in living animals suggest that these cells can continuously release analgesic substances in vivo after transplantation. In addition, a more robust analgesic potential of chromaffin-like cell grafts compared to that of the hPPE-transfected NT2 (human neuron-committed teratocarcinoma) cell grafts as reported in our previous studies was found (17), that is, a low number of chromaffin-like cells (1×10^5) produced similar analgesia effects to that of a high number of hPPE-transfected NT2 cells (1×10^7) in the same animal models. The duration of the analgesic effects produced by chromaffin-like cells was similar to that of the porcine chromaffin cells reported previously (16), with a time-dependent gradual decline in analgesic efficacy. Although we did not perform histological examination for the grafted cell fate in this study, these results suggest that host immune responses to the transplanted xenogeneic cells may occur. As demonstrated by our previous study (23), only few xenogeneic cells could be detected in the transplanted animals without immunosuppression, suggesting that administration of immunosuppressants is necessary to maintain long-term survival and long-lasting analgesic effects of xenogeneic cell grafts. Further studies are warranted to determine the survival rate, phenotypic stability, and secretion activity of chromaffin-like cells in vivo by using autologous cell transplantation in related animal pain models.

It is common knowledge that use of immunosuppressants can have severe side effects, including tumor formation, and should be avoided when possible. Transplantation of cells derived from individuals' autologous tissues into the same individuals would be safe and immunocompatible compared to xeno- and allotransplants. In practice, hMSCs can be derived from a patient's own tissues and not only could be prepared to become isogeneic through this cell reprogramming approach but also could avoid immunological rejection through autologous cell transplantation. Thus, autologous chromaffin-like cells could be the most desirable alternative to adrenal chromaffin cells for potential therapeutic purposes. Robust and long-lasting analgesic effects of autologous chromaffin-like cells are expected because these cells would be spared from immune responses, thereby improving the therapeutic efficacy of the transplanted cells.

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REFERENCES

1. Bés, J. C.; Tkaczuk, J.; Czech, K. A.; Tafani, M.; Bastide, R.; Caratero, C.; Pappas, G. D.; Lazorthes, Y. One-year chromaffin cell allograft survival in cancer patients with chronic pain: Morphological and functional evidence. Cell Transplant. 7(3):227–238; 1998.
2. Cogle, C. R.; Yachnis, A. T.; Laywell, E. D.; Zander, D. S.; Wingard, J. R.; Steindler, D. A.; Scott, E. W. Bone marrow transdifferentiation in brain after transplantation: A retrospective study. Lancet 363(9419):1432–1437; 2004.
3. Collas, P.; Håkelien, A. M. Teaching cells new tricks. Trends Biotechnol. 21:354-361; 2003.
4. Czech, K. A.; Ryan, J. W.; Sagen, J.; Pappas, G. D. The influence of xenotransplant immunogenicity and immunosuppression on host MHC expression in the rat CNS. Exp. Neurol. 147(1):66–83; 1997.
5. Håkelien, A. M.; Gaustad, K. G.; Collas, P. Modulation of cell fate using nuclear and cytoplasmic extracts. Methods Mol. Biol. 325:99–114; 2006.
6. Håkelien, A. M.; Gaustad, K. G.; Collas, P. Transient alteration of cell fate using a nuclear and cytoplasmic extract of an insulinoma cell line. Biochem. Biophys. Res. Commun. 316:834–841; 2004.
7. Håkelien, A. M.; Gaustad, K. G.; Taranger, C. K.; Skalhegg, B. S.; Kuntziger, T.; Collas, P. Long term in vitro, cell type specific genome wide reprogramming of gene expression. Exp. Cell Res. 309:32–47; 2005.
8. Håkelien, A. M.; Landsverk, H. B.; Robl, J. M.; Skalhegg, B. S.; Collas, P. Reprogramming fibroblasts to express T cell functions using cell extracts. Nat. Biotechnol. 20:460–466; 2002.
9. Hama, A.; Sagen, J. Alleviation of neuropathic pain symptoms by xenogeneic chromaffin cell grafts in the spinal subarachnoid space. Brain Res. 651:183–193; 1994.

10. Landsverk, H. B.; Håkelien, A. M.; Küntziger, T.; Robl, J. M.; Skålhegg, B. S.; Collas P. Reprogrammed gene expression in a somatic cell-free extract. EMBO Rep. 3(4):384–389; 2002.
11. Lazorthes, Y.; Bès, J. C.; Sagen, J.; Tafani, M.; Tkaczuk, J.; Sallerin, B.; Nahri, I.; Verdié, J. C.; Ohayon, E.; Caratero, C. Transplantation of human chromaffin cells for control of intractable cancer pain. Acta Neurochir. Suppl. 64:97–100; 1995.
12. Lazorthes, Y.; Bés, J-C.; Sol, J-C.; Pappas, G. D. Intrathecal chromaffin cell allograft for cancer pain. In: Burchiel, K., ed. Surgical Management of Pain. New York, NY: Thieme Medical Publishers Inc.; 2001:973–978.
13. Lazorthes, Y.; Sagen, J.; Sallerin, B.; Tkaczuk, J.; Duplan, H.; Sol, J. C.; Tafani, M.; Bés, J. C. Human chromaffin cell graft into the CSF for cancer pain management: A prospective phase II clinical study. Pain 87:19–32; 2000.
14. Liechty, K. W.; MacKenzie, T. C.; Shaaban, A. F.; Radu, A.; Moseley, A. M.; Deans, R.; Marshak, D. R.; Flake, A. W. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nat. Med. 6(11):1282–1286; 2000.
15. Livett, B. G.; Dean, D. M.; Whelan, L. G.; Udenfriend, S.; Rossier, J. Co-release of enkephalin and catecholamines from cultured adrenal chromaffin cells. Nature 289(5795):317–319; 1981.
16. Lu, Y.; Jing, R.; Yeomans, D. C.; Pappas, G. D. Porcine chromaffin cells, culture, and transplant for antinociceptive effects in rodents and primates. Neurol. Res. 26(7):707–712; 2004.
17. Lu, Y.; Sugaya, I.; Zhang, Z. J.; Qu, T. Y.; Pappas, G. D.; Wilson, S. P.; Sugaya, K. Antinociceptive effect of intrathecal transplantation of preproenkephlin encoding neural progenitor cells in rat. Annual Meeting of Chicago Chapter Society for Neurosciences, Chicago, IL, March 11, 2005.
18. Majumdar, M. K.; Thiede, M. A.; Mosca, J. D.; Moorman, M.; Gerson, S. L. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J. Cell. Physiol. 176(1):57–66; 1998.
19. Makino, S.; Fukuda, K.; Miyoshi, S.; Konishi, F.; Kodama, H.; Pan, J.; Sano, M.; Takahashi, T.; Hori, S.; Abe, H.; Hata, J.; Umezawa, A.; Ogawa, S. Cardiomyocytes can be generated from marrow stromal cells in vitro. J. Clin. Invest. 103(5):697–705; 1999.
20. Michalewicz, P.; Laurito, C. E.; Pappas, G. D.; Lu, Y.; Yeomans, D. C. Purification of adrenal chromaffin cells increases antinociceptive efficacy of xenotransplants without immunosuppression. Cell Transplant. 8(1):103–109; 1999.
21. Nagashima, H.; Fujimura, T.; Takahagi, Y.; Kurome, M.; Wako, N.; Ochiai, T.; Esaki, R.; Kano, K.; Saito, S.; Okabe, M.; Murakami, H. Development of efficient strategies for the production of genetically modified pigs. Theriogenology 59:95–106; 2003.
22. Onishi, A.; Iwamoto, M.; Akita, T.; Mikawa, S.; Takeda, K.; Awata, T.; Hanada, H.; Perry, A. C. Pig cloning by microinjection of fetal fibroblast nuclei. Science 289:1188–1190; 2000.
23. Ortega, J. D.; Sagen, J.; Pappas, G. D. Short-term immunosuppression enhances long-term survival of bovine chromaffin cell xenografts in rat CNS. Cell Transplant. 1(1):33–41; 1992.
24. Pappas, G. D. Transplantation of chromaffin cells for the treatment of chronic pain: Clinical, biochemical and morphological findings. In: Kanno, T.; Nakazato, Y.; Kumakura, K.; eds. The Adrenal Chromaffin Cells. Sapporo, Japan: Hokkaido University Press; 1998:343–351.
25. Pappas, G. D.; Lazorthes, Y.; Bés, J. C.; Tafani, M.; Winnie, A. P. Relief of intractable cancer pain by human chromaffin cell transplants: Experience at two medical centers. Neurol. Res. 19:71–77; 1997.
26. Pereira, R. F.; Halford, K. W.; O'Hara, M. D.; Leeper, D. B.; Sokolov, B. P.; Pollard, M. D.; Bagasra, O.; Prockop, D. J. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc. Natl. Acad. Sci. USA 92(11):4857–4861; 1995.
27. Petersen, B. E.; Bowen, W. C.; Patrene, K. D.; Mars, W. M.; Sullivan, A. K.; Murase, N.; Boggs, S. S.; Greenberger, J. S.; Goff, J. P. Bone marrow as a potential source of hepatic oval cells. Science 284(5417):1168–1170; 1999.
28. Pittenger, M. F.; Mackay, A. M.; Beck, S. C.; Jaiswal, R. K.; Douglas, R.; Mosca, J. D.; Moorman, M. A.; Simonetti, D. W.; Craig, S.; Marshak, D. R. Multilineage potential of adult human mesenchymal stem cells. Science 284 (5411):143–147; 1999.
29. Polakiewicz, R. D.; Behar, O. Z.; Comb, M. J.; Rosen, H. Regulation of proenkephalin expression in cultured skin mesenchymal cells. Mol. Endocrinol. 6:399–408; 1992.
30. Qu, T. Y.; Dong, X. J.; Sugaya, I.; Vaghani, A.; Pulido, J.; Sugaya, K. Bromodeoxyuridine increases multipotency of human bone marrow-derived stem cells. Restor. Neurol. Neurosci. 22(6):59–68; 2004.
31. Rosmaninho-Salgado, J.; Araújo, I. M.; Alvaro, A. R.; Mendes, A. F.; Ferreira, L.; Grouzmann, E.; Mota, A.; Duarte, E. P.; Cavadas, C. Regulation of catecholamine release and tyrosine hydroxylase in human adrenal chromaffin cells by interleukin-1beta: Role of neuropeptide Y and nitric oxide. J. Neurochem. 109(3):911–922; 2009.
32. Sagen, J.; Kemmler, J. E.; Wang, H. Adrenal medullary transplants increase spinal cord cerebrospinal fluid catecholamine levels and reduce pain sensitivity. J. Neurochem. 56(2):623–627; 1991.
33. Sagen, J.; Pappas, G. D.; Perlow, M. J. Adrenal medullary tissue transplants in the rat spinal cord reduce pain sensitivity. Brain Res. 384:189–194; 1986.
34. Sagen, J.; Pappas, G. D.; Pollard, H. B. Analgesia induced by isolated bovine chromaffin cells implanted in rat spinal cord. Proc. Natl. Acad. Sci. USA 83(19):7522–7526; 1986.
35. Sanchez-Ramos, J.; Song, S.; Cardozo-Pelaez, F.; Hazzi, C.; Stedeford, T.; Willing, A.; Freeman, T. B.; Saporta, S.; Janssen, W.; Patel, N.; Cooper, D. R.; Sanberg, P. R. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp. Neurol. 164(2):247–256; 2000.
36. Schulz, C.; Eisenhofer, G.; Lehnert, H. Principles of catecholamine biosynthesis, metabolism and release. Front. Horm. Res. 31:1–25; 2004.
37. Shi, G.; Ma, K.; Li, R.; Pappas, G. D.; Qu, T. Analgesic effects of chromaffin-like cells produced *in vitro* via cell reprogramming of human bone marrow-derived mesenchymal stem cells. 39th annual meeting of Society for Neurosciences, Chicago, IL, Oct. 17–21, 2009.
38. Shi, G.; Ma, K.; Li, R.; Qu, T.; Pappas G. D. Reprogramming human mesenchymal stem cells to achieve the phenotypic characteristics of adrenal chromaffin cells by using cellular extracts and cell fusion. 37th annual meeting of Society for Neurosciences, San Diego, CA, Nov. 3–7, 2007.
39. Shi, G.; Ma, K.; Pappas, G. D.; Qu, T. Phenotypic characteristics of hybrid cells produced by cell fusion of porcine adrenal

chromaffin cells with human mesenchymal stem cells: A preliminary study. Neurol. Res. 30(3):217–222; 2008.
40. Sol, J. C.; Sallerin, B.; Larrue, S.; Li, R. Y.; Jozan, S.; Tortosa, F.; Mascott, C.; Carraoue, F.; Tafani, M.; Lazorthes, Y. Intrathecal xenogeneic chromaffin cell grafts reduce nociceptive behavior in a rodent tonic pain model. Exp. Neurol. 186(2):198–211; 2004.
41. Sugaya, I.; Qu, T.; Sugaya, K.; Pappas, G. D. Genetically engineered human mesenchymal stem cells produce met-enkephalin at augmented higher levels in vitro. Cell Transplant. 15:225–230; 2006.
42. Unsicker, K. The trophic cocktail made by adrenal chromaffin cells. Exp. Neurol. 123(2):167–173; 1993.

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Research paper

A new method to effectively and rapidly generate neurons from SH-SY5Y cells

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HIGHLIGHTS

- CM-hNSCs significantly increased the neuronal percent of RA-pretreated SH-SY5Y cells.
- CM-hNSCs with RA significantly shorten the time of neuronal differentiation of SH-SY5Y cells.
- CM-hNSCs significantly inhibited the apoptosis of differentiated SH-SY5Y cells due to the presence of RA.
- CM-hNSCs significantly promote the length of neurite of RA-pretreated SH-SY5Y cells.

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ABSTRACT

It is well known that neurons differentiated from SH-SY5Y cells can serve as cell models for neuroscience research; i.e., neurotoxicity and tolerance to morphine in vitro. To differentiate SH-SY5Y cells into neurons, RA (retinoic acid) is commonly used to produce the inductive effect. However, the percentage of neuronal cells produced from SH-SY5Y cells is low, either from the use of RA treatment alone or from the combined application of RA and other chemicals. In the current study, we used CM-hNSCs (conditioned medium of human neural stem cells) as the combinational inducer with RA to prompt neuronal differentiation of SH-SY5Y cells. We found that neuronal differentiation was improved and that neurons were greatly increased in the differentiated SH-SY5Y cells using a combined treatment of CM-hNSCs and RA compared to RA treatment alone. The neuronal percentage was higher than 80% (about 88%) on the 3rd day and about 91% on the 7th day examined after a combined treatment with CM-hNSCs and RA. Cell maturation and neurite growth of these neuronal cells were also improved. In addition, the use of CM-hNSCs inhibited the apoptosis of RA-treated SH-SY5Y cells in culture. We are the first to report the use of CM-hNSCs in combination with RA to induce neuronal differentiation of RA-treated SH-SY5Y cells. Our method can rapidly and effectively promote the neuronal production of SH-SY5Y cells in culture conditions.

1. Introduction

Neurons differentiated from SH-SY5Y neuroblastoma cells by RA (retinoic acid) treatment are widely used as *in vitro* cell models in neuroscience research topics including neurotoxicity and tolerance to morphine [4,5,9,12,17,19,20]. SH-SY5Y is a human-derived cell line, with the capacity to expand in culture prior to differentiation. In the literature, RA is the most commonly implemented and best-characterized agent to differentiate SH-SY5Y cells into neurons in vitro. However, only about 20% of SH-SY5Y cells can be differentiated into neurons with RA treatment alone [15]. In addition, it takes time (an average of 7 days) to produce a neuronal differentiation of SH-SY5Y cells with RA treatment alone [5,16]. Recently, some investigators proposed that other chemicals (i.e., herbimycin A (herb-A), 12-*O*-tetradecanoyl-phorbol-13

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acetate (TPA), and dibutyryl cyclic AMP [dbcAMP]) or neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) with or without extra cellular matrix (ECM) gel, can further improve neuronal differentiation and maintain these conditions in cultures of RA-treated SH-SY5Y cells [3,6,10,15]. Cultivating SH-SY5Y cells in primary neuronal cultures or NSC (neural stem cell) culture medium with a B27 supplement has been shown to enhance neuronal differentiation with RA treatment [17]. However, the percentage of neuronal cells in the differentiated SH-SY5Y cells and the time needed to induce neuronal differentiation remain unsatisfactory under these combined conditions. Furthermore, these methods place an increased financial burden on experimental expenses. Thus, it is necessary to find an effective method to increase neuronal differentiation and production and at the same time to reduce the neuronal differentiation time of SH-SY5Y cells, thus making differentiated SH-SY5Y cells an ideal cell model.

NSCs (neural stem cells) have been successfully isolated from human brain tissue and can be expanded in vitro for an extended time in neural basal culture medium supplemented with B27 and mitotic factors bFGF (basic fibroblast growth factor) and EGF (epidermal growth factor) [18]. Human NSC (hNSC) lines are currently commercially available for research use. It has been reported that NSCs can secrete many neurotrophic factors, such as BDNF, NGF, GDNF (glial cell line-derived neurotrophic factor), and NT-3 (neurotrophin-3), as well as other soluble factors in culture conditions [2,8,13]. Thus, CM-NSCs (conditioned medium collected from hNSC cultures) containing these already known neurotrophic factors may also contain unknown factors released by hNSCs. In previous studies, our group successfully generated self-renewable functional NSC-like cells from hBM-MSCs (human bone marrow-derived mesenchymal stem cells) cultivated in CM-NSCs [7,14], demonstrating that CM-NSCs plays a crucial role in promoting the neuronal differentiation of these connective tissue cells. We hypothesized that CM-NSCs used to culture SH-SY5Y cells with an RA combination may increase neuronal differentiation and production of these cells.

2. Materials and methods

2.1. Collection of CM-hNSCs

hNSCs were purchased from Lonza (Walkersville, MD, USA). The detailed method for the growth and maintenance of undifferentiated hNSC spheroids and for collecting CM-hNSCs was performed according to the protocol we established previously [14]. In brief, hNSCs were cultured in a T-75 flask containing 15 ml complete culture medium including DMEM/F12 (Invitrogen, CA, USA), human recombinant epidermal growth factor (EGF; 20 ng/ml) and basic fibroblast growth factor (bFGF; 20 ng/ml) (R&D Systems, Minneapolis, MN, USA), B27 (serum-free medium supplements formulated to provide optimal growth condition for NSC expansion, 1: 50; Invitrogen), heparin (5 μg/ml; Sigma, St Louis, MO, USA), 2 mM L-glutamine, and an antibiotic–antimycotic mixture (1: 100; Invitrogen) at 37 °C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA, USA). Fifty percent of the culture medium was exchanged with fresh culture medium every 3 days. Larger neuro-spheroids were cut into small spheroids observed under a microscope (Olympus, Japan) every 2 weeks. At each medium change, CM-hNSCs were collected and filtered through a membrane with a 0.22 μm in diameter pore size (Millipore, Billerica, MA, USA). The filtered conditioned mediums were centrifuged at 1000 RPM × 10 min at RT (room temperature) and observed under a microscope to make sure there was no cell contamination.

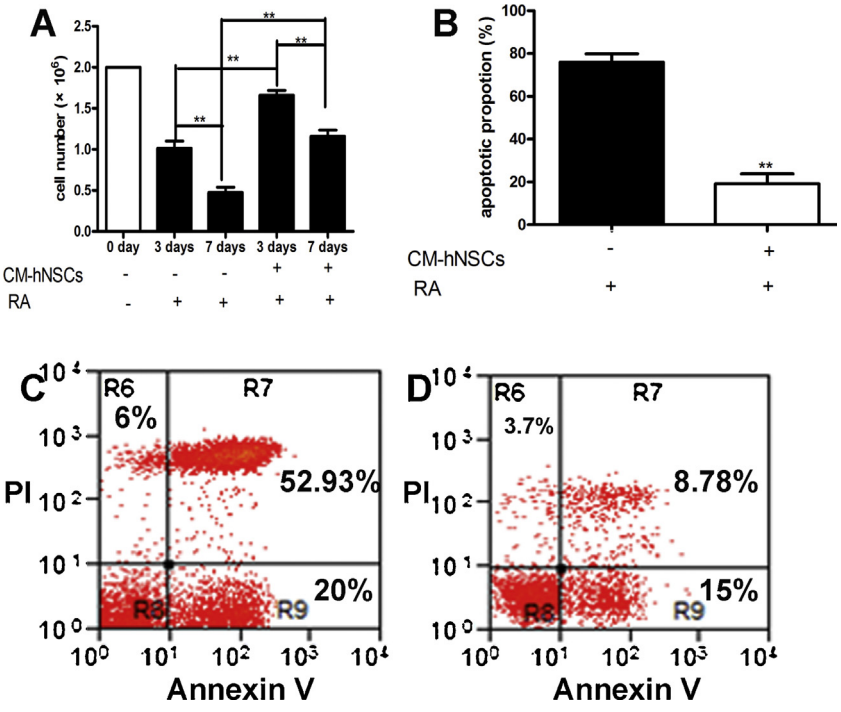

Fig. 1. CM-hNSCs significantly improved the survival of RA-treated SH-SY5Y cells.
(A) Bar graph showing that the cell number at the 3rd day was higher than that at the 7th day following RA treatment in the presence or absence of CM-hNSCs. However, note that the presence of CM-hNSCs significantly improved the cell survival rate, with a higher cell number than that in the absence of CM-hNSCs, at both of the 3rd and 7th day following RA treatment. One-way ANOVA, $^{**}P < 0.01$(B) Bar graph showing that the apoptotic percentage of RA-treated SH-SY5Y cells was significantly decreased in the presence of CM-hNSCs. Two-sample *t*-test, $^{**}P < 0.01$(C and D). The dot plots showing the representative flow cytometric results of PI/annexin V staining in the absence (C) and presence (D) of CM-hNSCs at the 7th day following differentiation of the RA-treated SH-SY5Y cells.

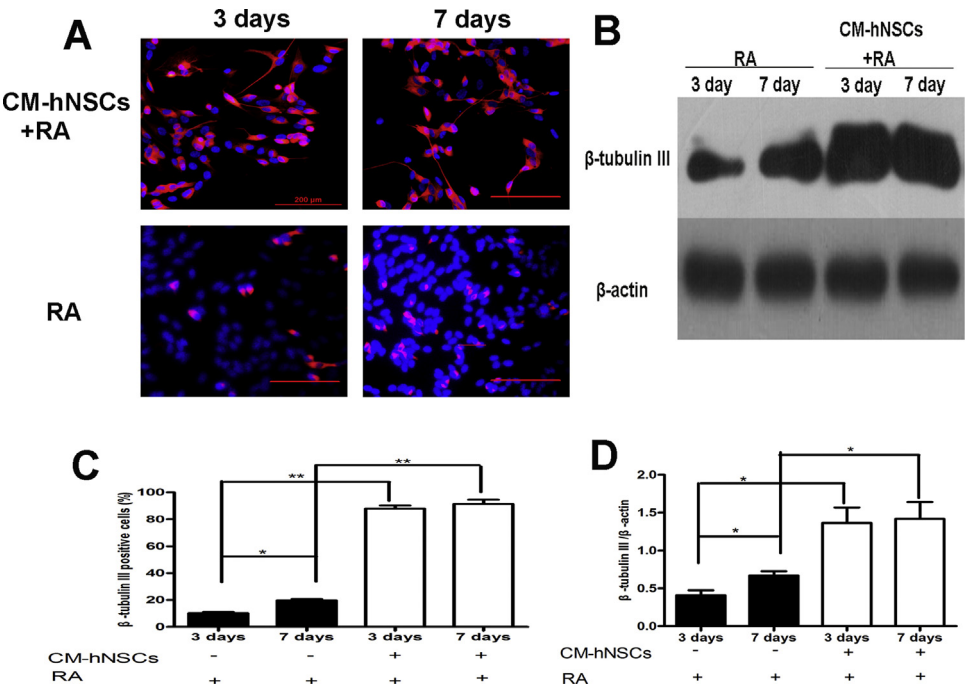

Fig. 2. CM-hNSCs significantly increased the neuronal production of RA-treated SH-SY5Y cells.
(A) Representative fluorescent immunocytochemical results showing β-tubulin III-positive cells (red) in the population of differentiated SH-SY5Y cells examined at the 3rd and 7th day of RA treatment in the presence and absence of CM-hNSCs. Bar: 200 μm.(B) Representative Western blot results showing the expression levels of β-tubulin III in the differentiated SH-SY5Y cells examined at the 3rd and 7th day of RA treatment in the presence and absence of CM-hNSCs.(C and D) Bar graph showing that when examined at the 3rd and 7th day following differentiation, a combined application of CM-hNSCs and RA significantly increased the number of β-tubulin III-positive cells (C) and the expression levels of β-tubulin III (D) in SH-SY5Y cells compared to RA treatment alone. One-way ANOVA, $^{**}P < 0.01$, $^{*}P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Neuronal differentiation of SH-SY5Y cells

SH-SY5Y cells were purchased from Sigma (St Louis, MO, USA). The cells were cultured in DMEM/F12 (Invitrogen), including 15% FBS, 2 mM L-glutamine, and an antibiotic–antimycotic mixture (1:100; Invitrogen) at 37 °C in a 5% CO_2 humidified incubation chamber. SH-SY5Y cells used for the experiment were controlled between passages 25 and 30. The cells (1×10^5) plated onto poly-lysine coated coverslips (10 mm in diameter) in 1 ml culture medium were used for immunocytochemical studies while the cells (2×10^6) seeded into 10 cm tissue culture dishes in 10 ml culture medium were used for Western Blot analysis, cell counting, and PI/annexin V staining. In total, four groups were divided. A & B: Cells were cultured in DMEM/F12 (Invitrogen) containing 3% FBS and 10 μM RA (Sigma) for 3 days (A) and 7 days (B) as control groups. C & D: The cells were cultured in CM-hNSCs containing 10 μM RA (Sigma) for 3 days (C) and 7 days (D) as experimental groups. Every day, the fresh medium was changed and each experiment was performed in duplicate.

2.3. PI (propidium iodide)/annexin V staining

After 7 days of differentiation, the cells were harvested from the tissue culture dishes by trypsin and prepared as a single cell suspension. We counted the number of differentiated cells by Countess™ Automated Cell Counter (Invitrogen) before performing PI/annexin V staining. The protocols for conducting PI/annexin V staining were according to the manufacturer's instructions (BD, 556,547). In brief, 1×10^6 cells were re-suspended into 1 ml 1× binding buffer containing 5 μl annexin V and 5 μl PI and incubated for 15 min in the dark. Prior to flow cytometry (Dako, Denmark), a supplement of 400 μl 1× binding buffer was added to each sample.

2.4. Immunocytochemistry

At the 3rd and 7th day of the differentiation in culture, cells on the coverslips were washed twice using PBS and fixed in 4% PFA (paraformaldehyde, Sigma) for 30 min at RT. After washing twice in PBS, cells were blocked in PBS containing 5% goat serum for 1 h at RT, followed by incubation with rabbit anti β-tubulin III (1:1000, Abcam) overnight at 4 °C. The second day, cells were incubated with TRITC-conjugated goat anti-rabbit IgG (1:200 Jackson ImmunoResearch Lab) for 2 h in the dark at RT after washing in PBS twice. Finally, cells were coverslipped with the medium for fluorescence with DAPI (Vector Labs) and viewed by fluorescence microscopy (Olympus, Japan). The number of positive cells stained by specific antibodies was counted at ×200 magnification. Data were averaged, expressed as the mean ± SEM, and compared between groups.

2.5. Western blot

The Western blot procedure was performed according to the protocol described in publication [4]. At the 3rd and 7th day of the differentiation, cells on the tissue culture dishes were washed twice in PBS, treated in 0.25% trypsin and EDTA for 1 min at 37 °C in a 5% CO_2 humidified incubation chamber and the suspending cells were collected. After that, cells were incubated in ice-cold RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 0.5% deoxycholic acid, 0.1% SDS, 1% Triton X-100, 2.5 mM EDTA) including protease inhibitors (1 mM, Beyotime, China) for 30 min at 4 °C. The cells were then centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was collected and protein concentration was measured using a BCA protein assay kit (Beyotime, China). Proteins were separated on 10% SDS polyacrylamide gels according to the molecular weight of the detection proteins and transferred onto PVDF membranes by electrophoresis. The membranes were blocked with 5% non-

fat milk in TBST (TBS containing 0.1% Tween-20) for 1 h at RT and incubated with rabbit anti-β-tubulin III (1:600, Abcam) and mouse anti-β-actin (1:1000, Santa Cruz) primary antibodies overnight at 4 °C, followed by staining with goat anti-rabbit and anti-mouse secondary antibodies conjugated to horseradish-peroxidase (HRP, Beyotime, China) for 1 h at RT. Finally, proteins were detected by chemiluminescence assay. Blots were repeated three times for every set of experiments. Bands were normalized to β-actin levels and the density of the bands were measured using Image J analysis software (NIH, USA).

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad). Data were expressed as means with ±SEM. A two-sample *t*-test was used to analyze the apoptotic percentage between groups. A one-way ANOVA was performed to analyze other data unless specifically stated otherwise. $P < 0.05$ was used as the significance level.

3. Results

3.1. CM-hNSCs significantly improved the survival of RA-treated SH-SY5Y cells

To confirm whether CM-hNSCs have a protective effect on the survival of RA-treated SH-SY5Y cells, we counted the cell number by a Countess™-automated cell counter after different treatments. We found that RA resulted in a decrease in the number of treated SH-SY5Y cells, however, the cell number was significantly higher in the presence of CM-hNSCs compared to those in the absence of CM-hNSCs (Fig. 1A, $^{**}p < 0.01$), suggesting that CM-hNSCs have a beneficial effect on the survival of RA-treated SH-SY5Y cells. To investigate whether the decreased number of RA-treated SH-SY5Y was attributable to cell apoptosis, we applied propidium iodide (PI) and annexin V staining for flow cytometry analysis. On the 7th day of RA treatment, the apoptotic percentage of differentiated SH-SY5Y cells in the presence of CM-hNSCs was about 19% ± 4.5% while the apoptotic percentage of differentiated SH-SY5Y cells in the absence of CM-hNSCs was about 76% ± 3.8% (Fig. 1B–D). Taken together, these data suggest that RA can lead to the apoptosis of treated SH-SY5Y cells whereas CM-hNSCs can greatly counteract the cell apoptosis resulting from RA treatment.

3.2. CM-hNSCs significantly increased the neuronal production of RA-differentiated SH-SY5Y cells

To assess the potential of RA-treated SH-SY5Y cells to differentiate into neurons in CM-hNSCs, we performed fluorescent immunocytochemical staining in cultured cells for the neuron-specific marker β–tubulin III. We observed that CM-hNSCs significantly increased the percentage of neuronal cells in RA-treated SH-SY5Y cells compared to that of neuronal cells in SH-SY5Y cells treated by RA alone (Fig. 2A and C, $^{**}P < 0.01$). In the presence of CM-hNSCs, neuronal percentage in differentiated SH-SY5Y cells was increased from 10% to 88% after 3 days of differentiation and from 20% to 91% after 7 days of differentiation compared to RA treatment alone. No significant difference was detected between the percentage of neuronal cells differentiated for 3 and 7 days in the presence of CM-hNSCs and RA, suggesting that CM-hNSCs have substantially lessened the time for neuronal differentiation of RA-treated SH-SY5Y cells. In addition, we observed that β–tubulin III-positive neurites were extensively long in the group using CM-hNSCs and RA treatment. However, very few β-tubulin III -positive neurites were detected in the group with RA treatment alone (Fig. 2A). To confirm the increased neuronal differentiation of RA-treated SH-SY5Y cells with CM-hNSCs, we further examined the protein levels of neuron-specific marker β-tubulin III by Western blot. Our results showed that the protein levels of β-tubulin III with CM-hNSCs and RA treatment were significantly higher than those with RA treatment alone (Fig. 2B and D, $^{*}P < 0.05$). Collectively, these data indicate that the combined application of CM-hNSCs and RA rapidly and effectively increased neuronal production of SH-SY5Y cells in culture compared to RA treatment alone.

4. Discussion

To date, the methodology to rapidly and effectively generate a good percentage of neuronal cells from SH-SY5Y cells for research purposes has been lacking although much effort has been expended, including the combined application of RA and other chemicals or neurotrophic factors, such as BDNF and NGF [3,6,10]. In the current studies, we demonstrated that the combined application of CM-hNSCs and RA in culture not only results in rapid and effective neuronal differentiation but also significantly increased the percentage of neuronal cells in the differentiated SH-SY5Y cells. In addition, CM-hNSCs demonstrated a beneficial effect on the survival of differentiated SH-SY5Y cells and significantly decreased the apoptotic portion of the RA-treated SH-SY5Y cells.

It is well known that SH-SY5Y cells, morphologically characterized by neuroblast-like and non-polarized cell bodies with few and truncated processes, can continuously proliferate in culture but lack neuronal markers prior to differentiation [6]. β-tubulin III is a neuron-specific marker in which levels increase with the rate of neuronal differentiation and maturation. Previous studies reported that SH-SY5Y cells can exhibit this property for neurons in molecular and protein levels at 7 or more days when cultured in low serum culture conditions with 10 μM RA supplements [16]. Our studies found that the percentage of β-tubulin III-positive cells in differentiated SH-SY5Y cells was only 10% on the 3rd day and about 20% on the 7th day when treated by RA alone (Fig. 2A and C). These data suggest that there is an increased neuronal differentiation of SH-SY5Y cells with the increased duration of RA treatment. However, the percentage of apoptotic cells was also found to be increased with an increased duration of RA treatment, as demonstrated in our studies (Fig. 1). In addition, we found few neurites or fibers of differentiated neurons at 3 or 7 days after RA treatment (Fig. 2A). These observations suggest that most neuronal cells resulting from RA-treated SH-SY5Y cells are immature. In contrast, the combined application of CM-hNSCs and RA in culture produced neurons with a round cell body and long processes; i.e., the morphological characteristics of typically mature neurons (Fig. 2A). Furthermore, the neuronal percentage detected from differentiated SH-SY5Y cells was as high as 80% at 3 days only and about 91% at 7 days after combined RA and CM-hNSC treatment (Fig. 2A and C). There was no significant difference in neuronal percentage between 3 and 7 days of RA and CM-hNSC treatment (Fig. 2A and C). These data suggest that 3 days is sufficient for neuronal production of SH-SY5Y cells cultured in CM-hNSCs and RA and our protocol significantly shortened the time that SH-SY5Y cells needed to produce neuronal differentiation. In addition, the increased number of neuronal cells in the combined treatment of CM-hNSCs and RA is confirmed by the augmented expression of β-tubulin III in those differentiated SH-SY5Y cells with Western blot assay (Fig. 2A and B).

It was reported that RA can activate the survival signal pathways of differentiated SH-SY5Y cells by phosphorylation of Akt at serine 473 [4,15]. In contrast to previous studies, our results showed that the total number of differentiated SH-SY5Y cells was significantly decreased following RA treatment. However, we also found that the number of RA-treated SH-SY5Y cells was significantly high in

the presence of CM-hNSCs compared to that in the absence of CM-hNSCs (Fig. 1A $^{**}P < 0.01$), suggesting that CM-hNSC has a beneficial effect on the survival of differentiated SH-SY5Y cells. A previous *in vivo* study by other investigators showed that CM-hNSCs can reduce the number of caspase 3-positive apoptotic profiles of spinal cord injuries [11]. Consistently, our results further confirmed that the decreased number of differentiated SH-SY5Y cells is due to apoptosis resulting from RA treatment, whereas CM-hNSCs can protect these RA-differentiated SH-SY5Y cells from apoptosis, as demonstrated by the PI/annexin V staining results (Fig. 1B, $^{**}P < 0.01$).

Neuronally differentiated SH-SY5Y cells are effective *in vitro* cell models for various neuroscience experiments that need to express specific markers for neurons such as β-tubulin III. However, there are other specific markers for neuronally differentiated SH-SY5Y cells used to confirm that these cell models are suitable for specific disease research. For example, RA-treated SH-SY5Y cells can express adult splicing forms of tau with neuronal localization as well as tyrosine hydroxylase (TH) [1,10], making these cells suitable for in vitro cellular models of Alzheimer's disease (AD) and Parkinson's disease (PD) research. In addition, they can also serve as a cell model for research in morphine tolerance, since the differentiated SH-SY5Y cells are able to express the opioid receptor μ [19]. It would be interesting to investigate the expression of these specific markers in neurons differentiated from SH-SY5Y cells under these culture conditions with a combination of RA and CM-hNSC applications.

Our studies have clearly shown that SH-SY5Y cells cultured in CM-hNSCs containing RA can effectively and rapidly differentiate into a number of neuronal cells. The CM-hNSCs used in culturing SH-SY5Y cells in the current studies are cell free, thus the possibility of neural cell contamination can be excluded. These results suggest that soluble neurotrophic factors released from hNSCs in the CM are important for the resulting neuronal production of SH-SY5Y cells. These soluble neurotrophic factors may include those already known; i.e., BDNF, NGF, GDNF, and NT-3 [2,8,13], as well as many other unknown soluble factors. Although the combined application in cultures of RA and primary neuronal cells or the use of NSC culture medium supplemented with B27 or BDNF or NGF can help improve the neuronal production of SH-SY5Y cells [3,6,10], the degree and extent of the increased neuronal percentage in these studies are still low compared to that produced using our protocol, suggesting that the combination of the known and unknown soluble factors and/or some unknown soluble factors in the CM-hNSCs may have significantly contributed to the increased neuronal differentiation and production of SH-SY5Y cells in culture. In the current preliminary studies, however, we did not perform experiments to focus on those unknown soluble factors that exist in CM-hNSCs. Further studies are warranted to identify those unknown soluble factors by global proteomic analysis of CM-hNSC contents in the future, which may help elucidate the mechanism of CM-hNSCs in the neuronal differentiation of SH-SY-5Y cells.

In conclusion, we are the first to demonstrate that a combined application of RA and CM-hNSCs can not only rapidly and effectively promote the neuronal differentiation of SH-SY5Y cells but can also greatly increase the percentage of neurons in the treated SH-SY5Y cells. CM-hNSCs has also a beneficial effect on the survival of the differentiated SH-SY5Y cells and can protect these cells from the apoptotic processes resulting from RA treatment.

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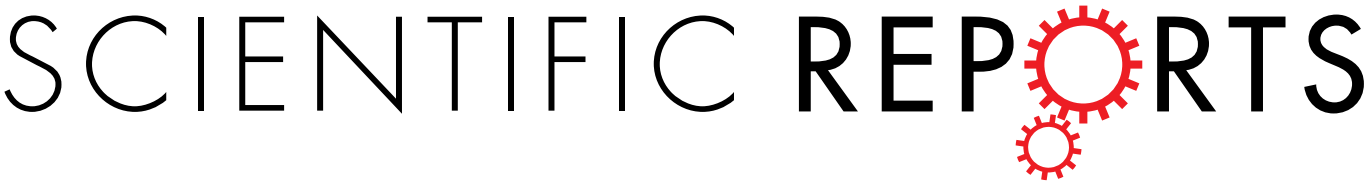
References

[1] L. Agholme, T. Lindstrom, K. Kagedal, J. Marcusson, M. Hallbeck, An in vitro model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons, J. Alzheimer's Dis. 20 (2010) 1069–1082.
[2] T.D. Aumann, R. Talaverón, E.R. Matarredona, R.R. de la Cruz, A.M. Pastor, Neural progenitor cell implants modulate vascular endothelial growth factor and brain-derived neurotrophic factor expression in rat axotomized neurons, PLoS One 8 (2013) e54519.
[3] J. Chen, B. Chattopadhyay, G. Venkatakrishnan, A.H. Ross, Nerve growth factor-induced differentiation of human neuroblastoma and neuroepithelioma cell lines, Cell Growth Differ. 1 (1990) 79–85.
[4] Y.-T. Cheung, W.K.-W. Lau, M.-S. Yu, C.S.-W. Lai, S.-C. Yeung, K.-F. So, R.C.-C. Chang, Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research, Neurotoxicology 30 (2009) 127–135.
[5] R. Constantinescu, A.T. Constantinescu, H. Reichmann, B. Janetzky, Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y, J. Neural. Transm. Suppl. (2007) 17–28.
[6] M. Encinas, M. Iglesias, Y. Liu, H. Wang, A. Muhaisen, V. Cena, C. Gallego, J.X. Comella, Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells, J. Neurochem. 75 (2000) 991–1003.
[7] L.E. Fox, J. Shen, K. Ma, Q. Liu, G. Shi, G.D. Pappas, T. Qu, J. Cheng, Membrane properties of neuron-like cells generated from adult human bone-marrow-derived mesenchymal stem cells, Stem Cells Dev. 19 (2010) 1831–1841.
[8] B. Hattiangady, A.K. Shetty, Neural stem cell grafting counteracts hippocampal injury-mediated impairments in mood, memory, and neurogenesis, Stem Cells Transl. Med. 1 (2012) 696–708.
[9] J. Kovalevich, D. Langford, Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology, Methods Mol. Biol. 1078 (2013) 9–21.
[10] T. Kume, Y. Kawato, F. Osakada, Y. Izumi, H. Katsuki, T. Nakagawa, S. Kaneko, T. Niidome, Y. Takada-Takatori, A. Akaike, Dibutyryl cyclic AMP induces differentiation of human neuroblastoma SH-SY5Y cells into a noradrenergic phenotype, Neurosci. Lett. 443 (2008) 199–203.
[11] P. Liang, J. Liu, J. Xiong, Q. Liu, J. Zhao, H. Liang, L. Zhao, H. Tang, Neural stem cell-conditioned medium protects neurons and promotes propriospinal neurons relay neural circuit reconnection after spinal cord injury, Cell Transplant. 23 (Suppl. 1) (2014) 45–56.
[12] F.M. Lopes, R. Schröder, M.L. Frota da Jr., A. Zanotto-Filho, C.B. Müller, R.T. Pires, G.D. Colpo, D.P. Gelain, J.C. Kapczinski, C. Mda Fernandes, F. Klamt, Comparison between proliferative and neuron-like SH-SY5 Y cells as an in vitro model for Parkinson disease studies, Brain Res. 1337 (2010) 85–94.
[13] P. Lu, L.L. Jones, E.Y. Snyder, M.H. Tuszynski, Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury, Exp. Neurol. 181 (2003) 115–129.
[14] K. Ma, L. Fox, G. Shi, J. Shen, Q. Liu, J.D. Pappas, J. Cheng, T. Qu, Generation of neural stem cell-like cells from bone marrow-derived human mesenchymal stem cells, Neurol. Res. 33 (2011) 1083–1093.
[15] P.N. Preis, H. Saya, L. Nadasdi, G. Hochhaus, V. Levin, W. Sadee, Neuronal cell differentiation of human neuroblastoma cells by retinoic acid plus herbimycin A, Cancer Res. 48 (1988) 6530–6534.
[16] J.-R. Sarkanen, J. Nykky, J. Siikanen, J. Selinummi, T. Ylikomi, T.O. Jalonen, Cholesterol supports the retinoic acid-induced synaptic vesicle formation in differentiating human SH-SY5Y neuroblastoma cells, J. Neurochem. 102 (2007) 1941–1952.
[17] C.L. Sayas, The neurite retraction induced by lysophosphatidic acid increases Alzheimer's disease-like tau phosphorylation, J. Biol. Chem. 274 (1999) 37046–37052.
[18] Y. Sun, S. Pollard, L. Conti, M. Toselli, G. Biella, G. Parkin, L. Willatt, A. Falk, E. Cattaneo, A. Smith, Long-term tripotent differentiation capacity of human neural stem (NS) cells in adherent culture, Mol. Cell. Neurosci. 38 (2008) 245–258.
[19] Z. Wang, W. Sadee, Tolerance to morphine at the mu-opioid receptor differentially induced by cAMP-dependent protein kinase activation and morphine, Eur. J. Pharmacol. 389 (2000) 165–171.
[20] V.C. Yu, W. Sadee, Efficacy and tolerance of narcotic analgesics at the mu opioid receptor in differentiated human neuroblastoma cells, J. Pharmacol. Exp. Ther. 245 (1988) 350–355.



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Mesenchymal Stem Cells Reversed Morphine Tolerance and Opioid-induced Hyperalgesia

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More than 240 million opioid prescriptions are dispensed annually to treat pain in the US. The use of opioids is commonly associated with opioid tolerance (OT) and opioid-induced hyperalgesia (OIH), which limit efficacy and compromise safety. The dearth of effective way to prevent or treat OT and OIH is a major medical challenge. We hypothesized that mesenchymal stem cells (MSCs) attenuate OT and OIH in rats and mice based on the understanding that MSCs possess remarkable anti-inflammatory properties and that both OT and chronic pain are associated with neuroinflammation in the spinal cord. We found that the development of OT and OIH was effectively prevented by either intravenous or intrathecal MSC transplantation (MSC-TP), which was performed before morphine treatment. Remarkably, established OT and OIH were significantly reversed by either intravenous or intrathecal MSCs when cells were transplanted after repeated morphine injections. The animals did not show any abnormality in vital organs or functions. Immunohistochemistry revealed that the treatments significantly reduced activation level of microglia and astrocytes in the spinal cord. We have thus demonstrated that MSC-TP promises to be a potentially safe and effective way to prevent and reverse two of the major problems of opioid therapy.

Chronic pain is a significant public health problem. It afflicts more than 100 million Americans and costs more than $635 billion annually[1,2]. Opioids, such as morphine, play an indispensable role in pain relief but are often associated with two major problems: opioid tolerance (OT) and opioid-induced hyperalgesia (OIH)[3–7]. OT is a physiological process where the body adjusts to a medication of frequent exposure and requires escalating doses to achieve the same effect. OIH is a phenomenon, in which individuals taking opioids to treat pain paradoxically develop an increased sensitivity to noxious stimuli. Both OT and OIH in animals have been validated in humans[8,9]. Nearly 50,000 people die every year of opioid overdose in the US, leading the Center for Disease Control and Prevention (CDC) to declare the problem an ongoing "national epidemic". These facts underscore an urgent need for finding effective therapies to treat pain and OT and to reduce the disastrous outcomes associated with opioid treatment. Distinct molecular mechanisms are indicated for the two closely related but different phenomena[9]. Neuroinflammation, mediated by immune cells and glial cells, appears to play a central role[10,11]. Opioids such as morphine can cause neuroinflammation[12] through acting on Toll-like receptor 4 on microglia and lead to development of OT[13]. Similarly, OIH is mediated by μ opioid receptor-dependent expression of P2X4 receptors on microglia and release of brain-derived neurotropic factor (BDNF)[14]. The P2X4-BDNF-TrkB pathway mediates microglia-to-neurons signaling and leads to sensitization of spinal lamina I neurons and OIH[14]. Thus, modulating neuroinflammation may prove to be an effective strategy to treat both OT and OIH.

We aim to develop a safe and efficacious therapy for OT and OIH in clinical practice. We chose to use MSCs because of their powerful paracrine functions, as shown in animal models of diseases such as traumatic brain injury[15], peripheral neuropathy[16,17], and neuropathic pain[18]. Immunomodulatory and anti-inflammatory effects of MSCs were related to neuroprotection, neuroregeneration, and neuroneuromodulation in these studies. For example, intravenous (IV) injection of human adipose-derived MSCs (hAD-MSCs) induced a significant reduction in mechanical allodynia and complete reversal of thermal hyperalgesia in a dose-dependent fashion in a

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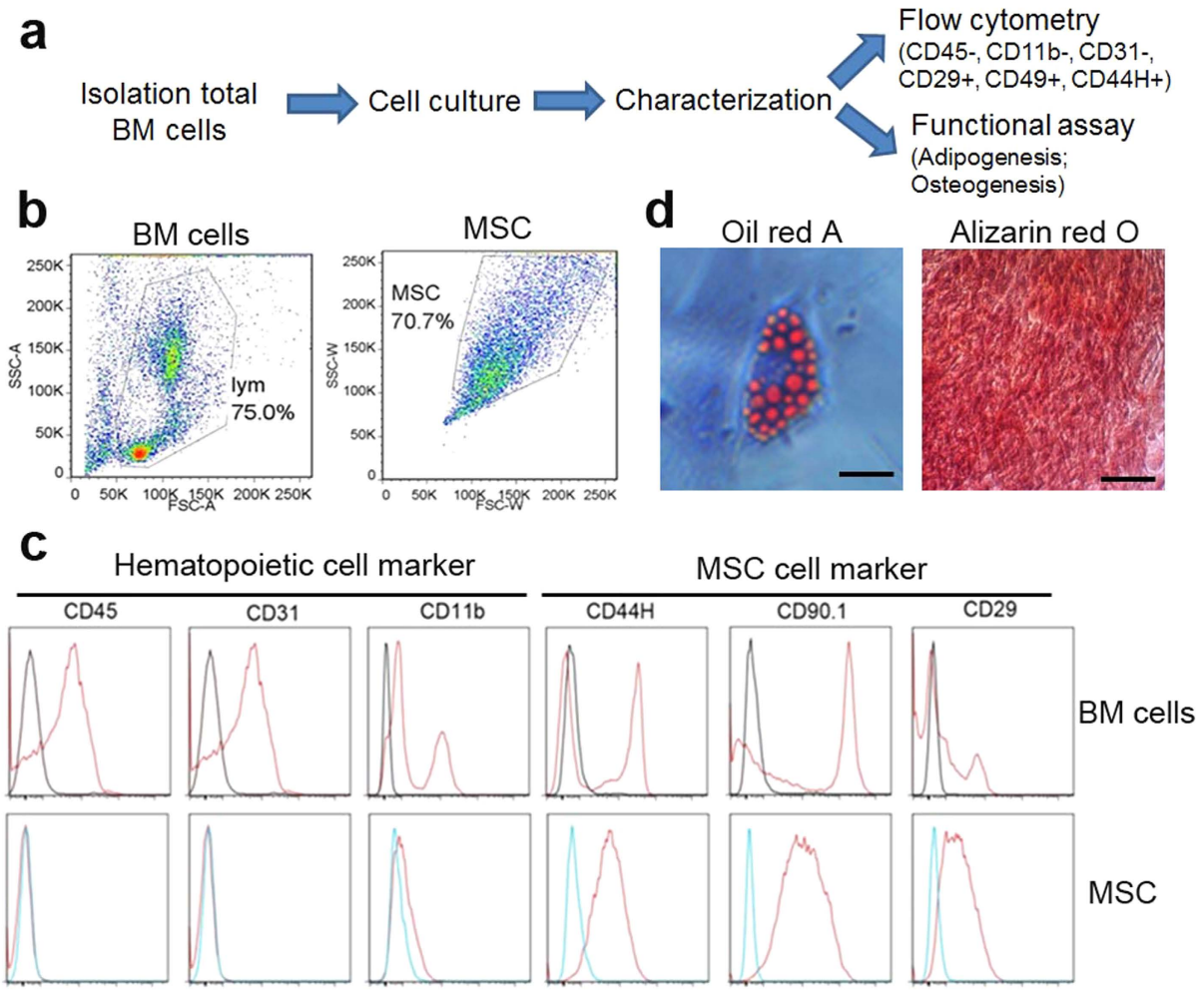

Figure 1. Isolation and characterization of MSCs from the rat bone marrow. (**a**) Experimental Scheme. (**b,c**) Flow cytometry data. (**b**) Different forward scatter (FCS) and side scatter (SSC) patterns between bone marrow (BM) cells (left), and bone marrow derived mesenchymal stem cells (MSCs) at passage 4 (right). (**c**) Cell surface markers (red) characteristic of hematopoietic cells and for MSCs respectively[68]. Unstained controls are indicated as blue. (**d**) MSCs were differentiated to adipose cells with lipid droplets accumulated in the cytoplasm stained with oil red (left) and osteoblast cells stained with Alizarin red (right) in respective media. These data represent three individual experiments.

mouse model of diabetic neuropathy[19]. The treatment decreased the level of IL-1β and increased IL-10 in the lesioned nerve and restored normal inducible nitric oxide synthase (iNOS) expression in the spinal cord. More recently, it was shown that IT rMSCs inhibited neuropathic pain via secretion of transforming growth factor beta (TGF-β)[20]. Thus, MSCs may release factors that promote tissue recovery through stimulating resident stem/progenitor cells, remodeling extracellular matrix, forming new blood vessels, and modulating immune functions[21–23].

We hypothesized that MSC transplantation (MSC-TP) attenuates chronic OT that is induced by long-term daily morphine injections. We further hypothesized that MSC-TP attenuates OIH that is developed as a consequence of chronic morphine injections. We tested these hypotheses by using intrathecal and intravenous routes of transplantation in rats and mice and studied the distribution of the transplanted cells and the level of activation of microglia and astrocytes in the spinal cord in response to morphine and MSC-TP.

Results

We first isolated MSCs from rat bone marrow and characterized the cells through flow cytometry (FACS) and induced differentiations. These cells showed morphological properties and cell markers characteristic of stem cells and differentiated into osteoblast cells and adipose cells in specific culture media (Fig. 1).

We then tested the preventive and therapeutic effects of intrathecal and intravenous MSC-TPs on OT, which was induced by daily morphine injections. Acute OT was induced after 3 days of daily injections. Administration of cumulative doses of morphine on day 4 produced a dose-response curve with a maximum effect dose of 18 mg/kg (MS: 24.52 ± 0.48, $n = 5$), which was significantly higher than that of the control group (8.0 mg/kg) (NS: 25.00 ± 0, $n = 3$) (Supplementary Fig. 1a; $P = 0.01$). Chronic OT was induced by daily morphine injections

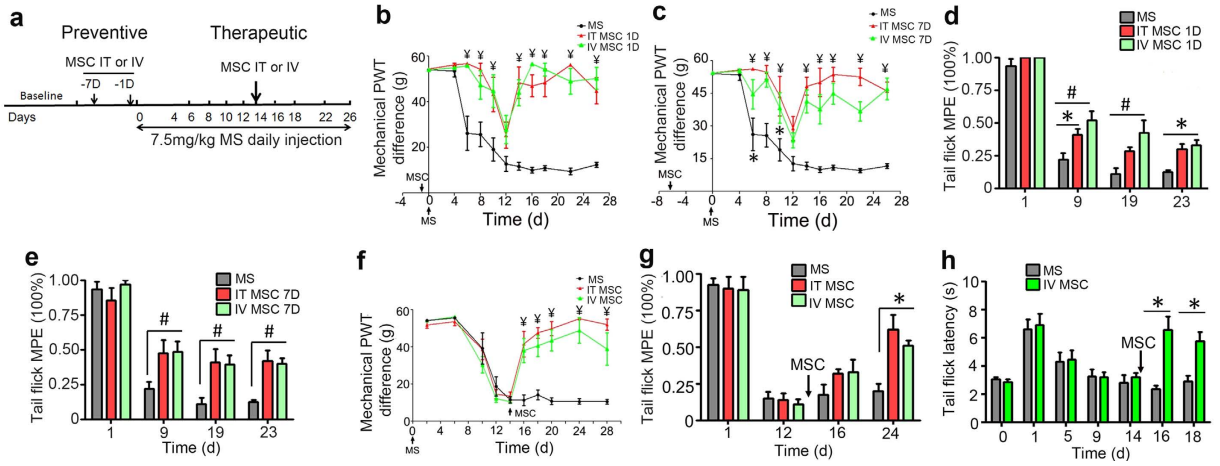

Figure 2. Preventive and Therapeutic effects of MSC transplantation (MSC-TP) on opioid tolerance (OT). (**a**) Experimental scheme. MSC-TP was performed either intravenously (IV) or intrathecally (IT) 1 day (**b,d**) or 7 days (**c,e**) before the initiation of daily MS injections. Pain-like behaviors in rats were assessed by von Frey filament (**b,c**) and tail flick tests (**d,e**). ($^{*}P < 0.05$, $^{\#}P < 0.01$, $^{¥}P < 0.001$ compared with the MS group n = 6 in each group). OT was induced by repeated daily MS injections at 7.5 mg/kg in rats (**f,g**) or 10 mg/kg in mice (**h**). MSC-TP was administrated at Day 14 when OT had fully developed. Pain-like behavior was evaluated by von Frey filament in rats (**f**) and tail flick tests in rats (**g**) and mice (**h**). ($^{*}P < 0.05$, $^{\#}P < 0.01$, $^{¥}P < 0.001$ compared with the same day MS group; n = 6 in MS group; n = 9 in IT and IV groups). Data: mean ± s.e. IT: intrathecal; IV: intravenous. MS, morphine sulfate; PWT, paw withdrawal threshold.

for 3 to 4 weeks and evaluated by measuring two sets of paw withdrawal thresholds (PWTs) to mechanical and thermal stimulation. The first were measured before daily morphine injection and the second were measured 50 min after the injection. The differences between the two sets reflect the level of tolerance. A large difference indicates no or low tolerance while a small difference indicates high tolerance. The differences decreased gradually and significantly after 7 days of morphine treatment, reached a minimal difference at day 12, and maintained a small difference thereafter (Supplementary Fig. 1b, $df = 7$, $F = 45.97$; $P < 0.0001$; $n = 8$). OT was further evaluated by the tail flick test. Maximum possible effect (MPE) of morphine was used to indicate tolerance; high MPE (%) indicates low or no tolerance while low MPE indicates high tolerance. MPE was significantly reduced after 7 days of morphine treatment, reaching ~10% of the baseline value by day 12 (Supplementary Fig. 1c; $df = 6$, $F = 47.34$; $P < 0.0001$; $n = 12$).

Intrathecal or intravenous MSC-TP did not cause any behavioral change in normal rats (Supplementary Fig. 1e,f, $P > 0.05$, $n = 6$ for NS, $n = 8$ for MSC). In contrast, it significantly and consistently attenuated the development of OT. Both intrathecal and intravenous MSCs were remarkably effective (Fig. 2a–h). A one-time transplantation significantly mitigated OT for the whole course of the experiments of up to 26 days (Fig. 2b,c). The effects were almost identical when the transplantation was performed one day or 7 days before morphine treatment. These experiments were repeated separately by two groups of experimenters who were blinded to the treatments (Supplementary Fig. 2). The effects were further evaluated by the tail flick tests (Fig. 2d,e. Figure 2d, $df = 3$, $F = 202.6$; $P < 0.0001$, $n = 6$ for each group; Fig. 2e: $df = 3$, $F = 131.7$; $P < 0.0001$, $n = 6$ for each group). Consistent with the paw withdrawal experiments, MSCs significantly increased the MPE (%) regardless of the route and time of the transplantation, although to a lesser extent (Fig. 2d,e).

MSC-TP significantly reversed established OT when it was performed 14 days after daily morphine treatment (Fig. 2f–h. Figure 2f: $df = 9$, $F = 40.13$; $P < 0.0001$, $n = 9$ for IV or IT MSC; MS $n = 6$; Fig. 2g: $df = 3$, $F = 139.3$; $P < 0.0001$, $n = 9$ for IV or IT MSC; MS $n = 6$; Fig. 2h: $df = 6$, $F = 14.95$; $P < 0.0001$, MSC $n = 8$; MS $n = 6$). Morphine-induced OT reached its peak at day 12 of daily morphine injections in both the mechanical and thermal tests (Fig. 2f–h). Both intrathecal and intravenous MSCs significantly and consistently restored the sensitivity to morphine. This therapeutic effect took place rather rapidly and lasted for the whole course of the experiment to day 28 with no sign of waning. Within 2 days of transplantation, the PWT differences increased significantly in both transplantation groups, compared to the control group ($P < 0.001$). Similarly, intrathecal or intravenous MSCs significantly increased tail flick MPE from below 15% to above 30% on day 16 and above 50% on day 24 (Fig. 2g) ($P < 0.05$). The therapeutic effects were further tested in mice. Intravenous transplantation of MSCs significantly attenuated OT. Compared to the control group, the mean tail flick latencies were significantly increased in the transplantation group (Fig. 2h). Consistent with the rat experiments, this effect took place within 2 days of the transplantation.

Next, we tested the preventive and therapeutic effects of MSCs on OIH, which was also induced by daily morphine injections. OIH was reflected by the progressive decline of PWTs from the baseline values established 3 days before morphine treatment (Supplementary Fig. 1d, $df = 17$, $F = 17.89$; $P < 0.0001$. MS $n = 1$; NS $n = 8$). The decline took place over a course of 5–7 days ($P < 0.001$) and the hyperalgesia status persisted even after the cessation of daily morphine injections. Such decline was not seen in the control group, which received daily saline

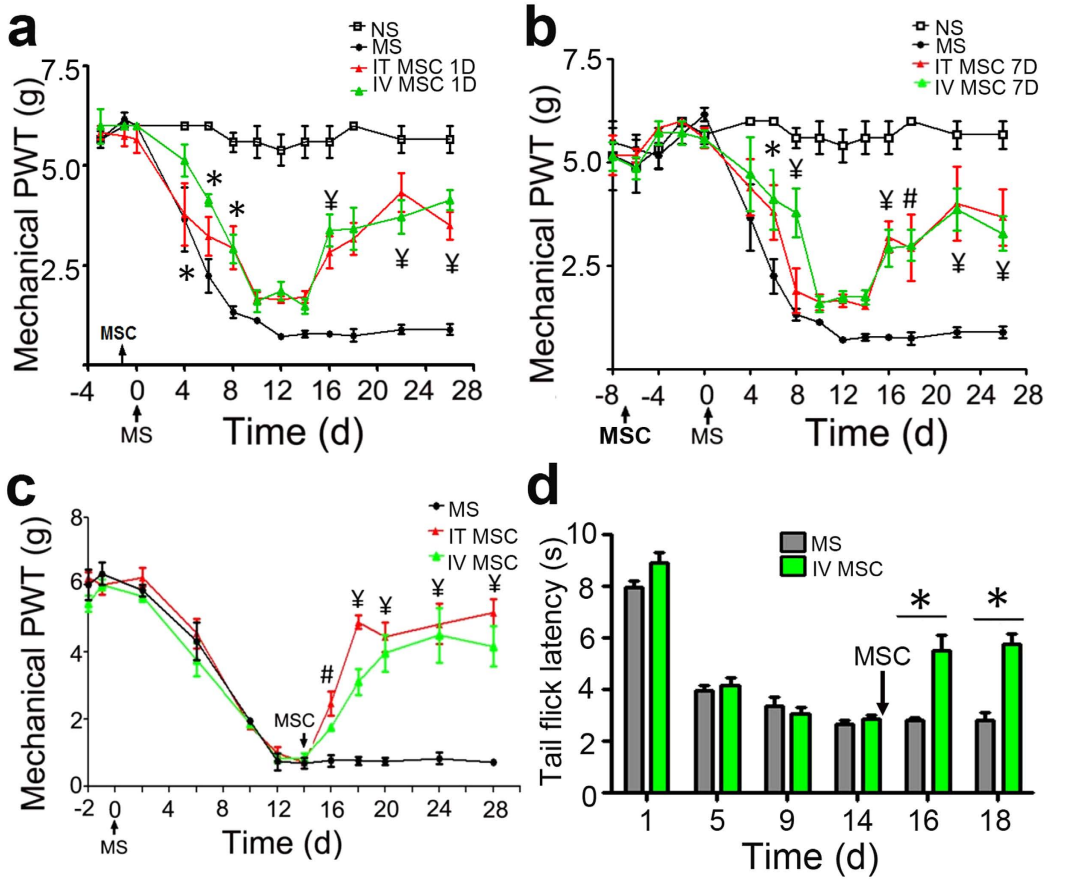

Figure 3. Preventive and therapeutic effects of MSC transplantation (MSC-TP) on opioid-induced hyperalgesia (OIH). MSC-TP was performed intrathecally (IT) or intravenously (IV) either 1 day (**a**) or 7 days (**b**) before the initiation of daily MS injections. MSC-TP of both routes significantly alleviated the morphine-induced reduction of PWTs, indicating preventive effects on OIH. (*$P < 0.05$, ¥$P < 0.001$ compared with the same day MS group; n = 6–8,). OIH was induced by daily MS injections of 7.5 mg/kg in rats (**c**) or 10 mg/kg in mice (**d**). MSC-TP was performed either intrathecally (IT) or intravenously (IV) at Day 14 in rats (**c**) or mice (**d**). Pain-like behavior was assessed by von Frey filament (**c**) or tail flick tests (**d**). MSC-TP of both routes consistently and significantly increased the mean PWT or the mean tail flick latency, indicating reversal of mechanical allodynia and thermal hyperalgesia in both rats and mice. *$P < 0.05$ compare to MS group (n = 5-8). Data: mean ± s.e. IT, intrathecal; IV, intravenous; MS, morphine sulfate; MSC-TP, MSC transplantation; NS: normal saline; PWT, paw withdrawal threshold.

injections ($P > 0.05$). Intrathecal or intravenous MSC-TP (5×10^5) substantially prevented the development of OIH (Fig. 3). The effects were long-lasting with no sign of waning over time. Consistent results were observed whether the transplantation was performed one day or seven days before morphine treatment. These results were replicated by two groups of experimenter who were blinded to the treatments. To test the therapeutic effects, MSC-TP was performed once OIH had fully developed. The transplantation effectively and rapidly reversed OIH. This effect lasted for the whole duration of the experiments (Fig. 3c, df = 11, F = 116.5; $P < 0.0001$, n = 6 for MS; n = 7 for IT or IV MSC). The therapeutic effect was further tested in mice (Fig. 3d, df = 5, F = 75.82; $P < 0.0001$. MS n = 6; MSC n = 8). Intravenous MSC-TP at day 14 of daily morphine treatment significantly increased the mean tail flick latency ($P < 0.001$).

All animals survived the entire course of the experiments up to 68 days and had normal locomotion, food and fluid intake, body weight gain, and biochemical parameters for liver and kidney functions (Supplementary Fig. 3, $P > 0.05$. NS group: n = 6, MS group n = 11, MS/MSC group n = 12). Histopathology examination at necropsy did not reveal any abnormality in any major organs. Dil labeled MSCs in red were successfully traced to the surface of the spinal cord and the dorsal root ganglia (DRG) after intrathecal transplantation (Fig. 4a–d). Double staining of Dil and DAPI (nucleus) confirmed viable MSCs on the dorsal side of the spinal cord and the DRGs at various time intervals. We did not find any Dil labeled MSCs in any of these tissues after intravenous transplantation.

Expressions of IBA-1 in microglia (green) and GFAP in astrocytes (red) in the spinal cord dorsal horn underwent significant changes in response to daily morphine injections and MSC-TP (Fig. 4e–h). The IBA-1 immuno-reactivity increased and the morphology of IBA-1 positive cells changed from ramified shape to an amoeboid shape in response to morphine treatment, evaluated at day 22 (Fig. 4f,g, control vs MS: 9.4 ± 2.0 vs 13.8 ± 4.6, $P < 0.05$, n = 12 for each group). MSC-TP largely restored the morphology to its resting state and

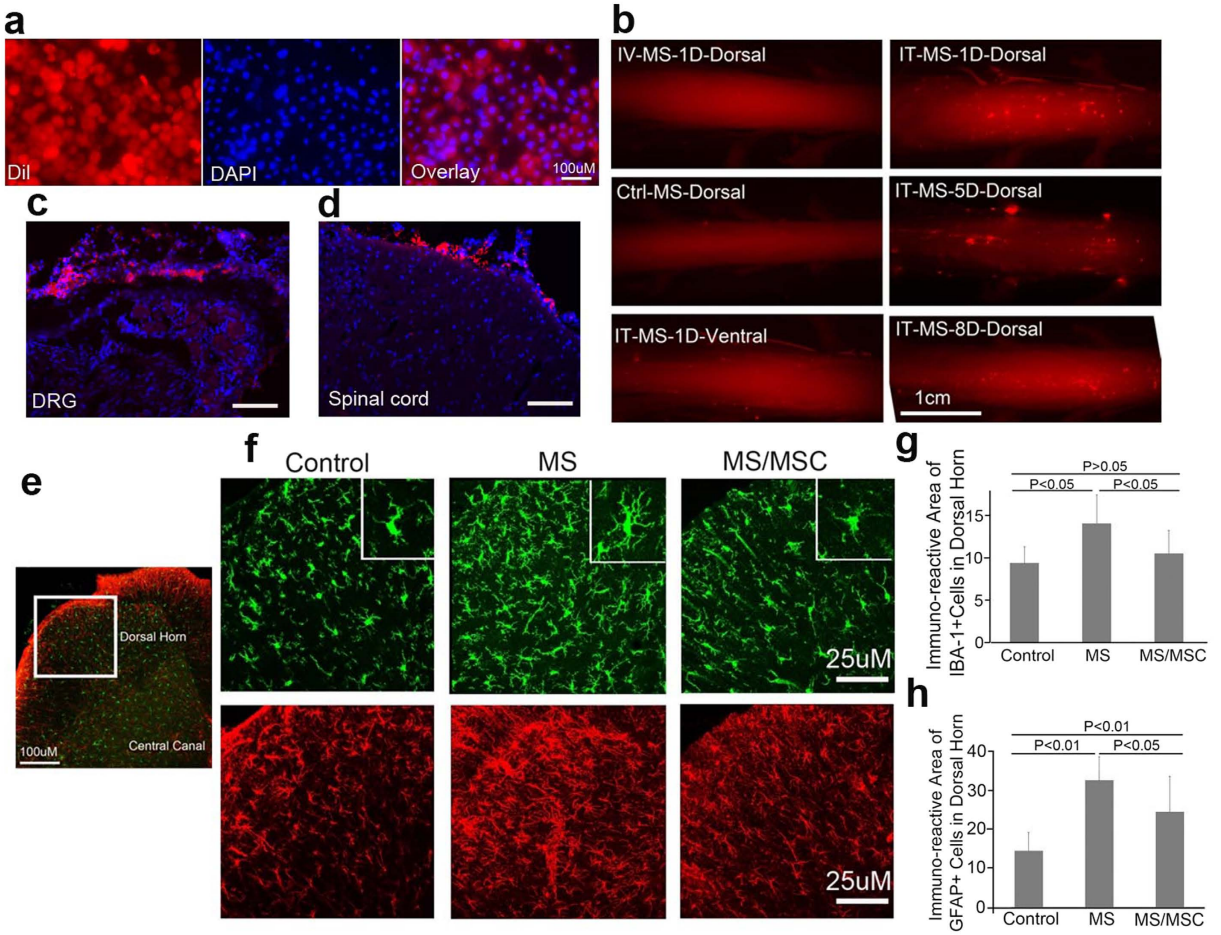

Figure 4. Tracing of transplanted MSCs and changes of microglia and astrocytes. (**a–d**) Fates of MSCs after intrathecal and intravenous transplantations. **(a)** Microscopy of labeled MSCs before injection. MSCs were labeled with Dil dye (red, left) and DAPI for nucleus (blue, middle). Double labeling of MSCs is shown on the right. (**b**) Microscopy pictures of the lumbar area of the spinal cord, demonstrating MSCs residing in the pia mater of the dorsal side of the spinal cord, as well as in the surrounding of the DRGs, 1 to 8 days after transplantation. Cross section of the DRG (**c**) and the spinal cord (**d**) showing MSCs residing in the pia mater of the spinal cord and DRG 1 day after MSC-TP. Scale bar: 50 um (**a**), 1 cm (**b**), 50 um (**c,d**). **(e–h)** MSC-TP attenuated activation of microglia and astrocytes. (**e**) Area of focus in the spinal cord dorsal horn in rats. Scale bar: 100 um. (**f**) Immunostaining of IBA-1(green) and GFAP (red) in the dorsal horn. Compared to controls, daily MS injections activated microglial cells (MS, top middle) and astrocyte (MS, bottom middle). MSC-TP attenuated this process (MS/MSC). Inserted boxes (top) show zoomed out individual microglia. Scale bar: 25 μm. Quantification results (mean ± s.e.) of immune-reactive areas of IBA-1 (**g**) and GFAP (**h**). n = 12 for each group. IT, intrathecal; IV, intravenous; MS: morphine sulfate; MSC-TP, MSC transplantation.

decreased the IBA-1 immuno-reactivity to a level that was not significantly different from the control (Fig. 4f,g, MS vs MS/MSC: 13.8 ± 4.6 vs 10.5 ± 2.7; $P < 0.05$; control vs MS/MSC: 9.4 ± 2.0 vs 10.5 ± 2.7, $P > 0.05$, n = 12 for each group). In addition, the GFAP immuno-reactivity was significantly increased after morphine treatment and was substantially reduced in the transplantation group (Fig. 4f,h, Control vs MS: 14.4 ± 4 vs 33.7 ± 9.9, $P < 0.01$; MS vs MS/MSC: 33.7 ± 9.9 vs 24.4 ± 9.1, $P < 0.05$; control vs MS/MSC: 14.4 ± 4 vs 24.4 ± 9.1, $P < 0.01$, n = 12 for each group).

Discussion

The search for effective preventive and therapeutic strategies to counteract OT and OIH has been invigorated by the gravity of the profound negative impacts of OT and OIH. Here we for the first time report a powerful anti-tolerance effect of MSC-TP. Both intrathecal and intravenous MSC-TPs effectively attenuated the development of OT when performed before the initiation of chronic daily morphine injections in rats. MSC-TP almost completely reversed chronic OT when performed after OT had been established, regardless of the route of transplantation. These findings were consistent in both rats and mice. Thus, we have provided several lines of evidence that MSC-TP is a promising preventive and therapeutic therapy for OT with great potentials for clinical translation.

We chose to focus on chronic OT, rather than acute OT, because it resembles more closely to clinical practice. In addition to the traditional approach to assessing OT by demonstrating a hallmark rightward shift in the agonist dose-response curve after 3 to 5 days of daily morphine administration (Supplementary Fig. 1a), we introduced a new paradigm to investigate chronic OT, which was induced by daily morphine injections for up to four weeks (Supplementary Fig. 1b–d). Preemptive MSC-TP significantly and persistently attenuated the development of OT (Fig. 2a–e). The results from two groups of investigators were strikingly consistent. The first group performed intrathecal MSC-TP and found a significant anti-tolerance effect (Supplementary Fig. 2). The second group further performed both intrathecal and intravenous MSC-TPs and confirmed the anti-tolerance effects by both routes of transplantation (Fig. 2a–e). In addition, we tested MSC-TP at two time points (1 day and 7 days) before morphine treatment and observed identical anti-tolerance effects (Fig. 2b,c). These data clearly indicate that MSC-TP, either intrathecally or intravenously, could effectively prevent the development of OT. In addition to the preventive effect, MSC-TP effectively reversed established OT and restored sensitivity to morphine (Fig. 2a,f–h). This finding is important because it suggests that the therapeutic effect may be applicable to an increasingly large population of patients with ongoing OT due to chronic use of opioids for a variety of cancer and non-cancer pain conditions. It is well known that analgesic tolerance is commonly developed but tolerance to opioid adverse effects, such as respiratory depression and constipation, does not readily develop. This differential tolerance is one of the most common reasons patients suffer from detrimental consequences including overdose and death when dose escalation is required to overcome analgesic tolerance. Managing this population is a daunting challenge even to well-trained pain specialists. MSC-TP promises to emerge as an effective therapy for OT. Its clinical translation may have a profound impact on improving the safety and efficacy of opioid therapy and reducing opioid overdose and death.

A second important finding of this work is the remarkable preventive and therapeutic effects of MSC-TP on OIH. We found a remarkable anti-hyperalgesia effect of MSC-TP in rats and mice. Similarly, the two groups of investigators independently showed consistent results and came to the same conclusions. The first group tested intrathecal MSC-TP and demonstrated a significant attenuation of the development of OIH. The second group tested both intrathecal and intravenous MSC-TPs and confirmed the preventive and therapeutic effects on OIH (Fig. 3). OIH and OT are related but distinct biological phenomena and clinical entities[24]. There is convincing evidence to clinically differentiate the two entities[9]. Dose escalation is required to overcome OT but such a strategy only further exacerbates OIH. In contrast, MSC-TP attenuated both OT and OIH.

Interestingly, intravenous MSC-TP resulted in similar degrees of anti-tolerance and anti-hyperalgesia effects compared to intrathecal MSC-TP (Figs 2 and 3). The former is clinically advantageous compared to the latter. However, since MSCs transplanted by this route are largely trapped in the lungs[25–27] and may be injured by activation of complements[28], we expected a short, if any, duration of the therapeutic effects. Surprisingly, both routes of MSC-TP achieved long-lasting preventive and therapeutic effects. This finding is clinically important and mechanistically intriguing. It not only indicates a convenient route of clinical application but also suggests systemic mechanisms of action. It doesn't seem to be necessary to place the cells in close proximity to the spinal cord (Fig. 4a–d). MSCs likely exert anti-tolerance and anti-hyperalgesia effects through their powerful paracrine function, regulating the sensitivity to noxious stimulation and opioid medications through modulation of immune and inflammatory processes in the peripheral and central nervous systems.

Microglial activation in the spinal cord plays a prominent role in the development of both OT and OIH[14,29]. Attenuating this process by MSCs is a plausible explanation for the observed effects. Indeed, microglial activation, induced by daily morphine injections, was significantly attenuated by MSC-TP (Fig. 4e–g). Also notable was the upregulation of GFAP expression after morphine treatment (Fig. 4e,f,h). MSC-TP partially and significantly restored GFAP expression. These data are consistent with the reports that chronic morphine injection activated spinal and cortical glia cells[30,31]. Morphine tolerance and hyperalgesia/allodynia have been associated with spinal microglial and astroglial activation[32]. Selective activation of an astrocyte JNK pathway after the stimulation of neuronal μ-opioid receptor (MOR) appears to mediate astrocyte-neuron signaling and contribute to OIH[33]. Inhibition of spinal glial activation by fluorocitrate, a nonselective metabolic inhibitor of astrocytes, partially reversed the development of morphine tolerance in rats[31]. These observations support the notion that both immune cells (microglia) and glial cells (astrocytes) are involved in the development of OT and OIH. MSC-TP may have achieved its therapeutic effects through acting on these cells. In addition, MSCs may modulate other cell types in the innate and adaptive arms of the immune system. For example, MSCs shifted the cytokine secretion profile of dendritic cells, naïve and effector T cells [T helper 1 (TH1) and 2 (TH2)], and natural killer cells to a more anti-inflammatory phenotype[34]. Undoubtedly, our current mechanistic understanding of the MSC therapy is in its infancy. It is important to appreciate its complexity and resist the temptation of attributing the therapeutic effects to a single molecular signaling pathway because MSCs may regulate immune cells, glial cells, and neurons by mechanisms that include both direct cell-to-cell contacts and release of a multitude of soluble factors.

Our data suggest that MSC-TP is safe and practical. All animals survived the whole experiments up to 68 days and maintained normal locomotion, food and fluid intake, body weight gain, and liver and renal function parameters (Supplementary Fig. 3). Histology at necropsy did not reveal any abnormality in any major part of the body. Thus, there was no evidence of toxicity even with long-term experiments up to 68 days. The intrathecally transplanted cells may have survived and maintained function *in vivo* for at least 34 days (Figs 2 and 3). This is consistent with our finding that viable MSCs were found in the pia mater of the spinal cord (Fig. 4). The long survival and long-lasting effects of MSCs are particularly important in clinical applications. Several factors may have contributed to this success. We used MSCs from the bone marrow in an early passage (passage #4) and the cells are essentially non-immunogenic[34–36]. A recent study used cells after 16 passages and failed to demonstrate any analgesic or anti-inflammatory effects[11]. Applications of human MSCs are being explored extensively[37] via multiple clinical trials on spinal cord injury[38,39], cardiovascular disease[40,41], Parkinson's disease[42], and diabetes[43]. Consistent with our results of xenogeneic transplantation from rats to mice (Figs 2 and 3), immune rejection has

not been a major concern because MSCs are immune-privileged due to their absent or low expression of major histocompatibility complex class II (MHC-II) and other co-stimulatory molecules[44]. Human MSCs are viable in tissues for months after systemic administration in sheep[45]. MSCs are known to have a strong immunosuppressive property and have been used successfully in autologous as well as allogeneic MSC-TP without pharmacological immunosuppression[46]. This unique capacity is being utilized in combating autoimmune diseases in clinical trials[47]. In addition, it is well accepted that MSCs have extremely low risk of tumorigenicity[48]; MSCs could actually inhibit tumor growth[49]. Clinical studies have convincingly demonstrated that direct injection of MSCs does not produce unwanted side effects and is well tolerated and safe[50,51].

In summary, we report a powerful anti-tolerance effect and a remarkable anti-hyperalgesia effect of MSC-TP in rats and mice. These effects were consistently observed by two groups of investigators independently. Both the intrathecal and intravenous routes of transplantation were effective. Intrathecally transplanted cells homed in the pia mater of the spinal cord and the DRGs and appeared to have maintained long-term viability. The animals showed normal vital functions without any trace of toxicity. The inhibitory effects of MSCs on microglia and astrocytes appeared to be related to the anti-tolerance and anti-hyperalgesia functions. It may be tempting to uncover a specific molecular or cellular mechanism of MSC action. However, it is most likely that multiple mechanisms are involved. MSCs may regulate immune cells and neurons by mechanisms that include both direct cell contact and release of soluble factors such as interleukin 10 (IL-10), leukemia inhibitory factor (LIF), and transforming growth factor (TGF) through a paracrine mechanism[52,53]. Activation of opioid receptors by MSCs may also contribute[54]. Collectively, we have demonstrated for the first time that MSC-TP promises to be an innovative, safe, efficacious, and cost-effective therapy to prevent and treat OT and OIH. This emerging therapy has enormous potential to profoundly impact clinical practice. It may improve the efficacy of opioid therapy, reduce the risk of opioid overdose, and save lives.

Methods

The research protocols were approved by the Cleveland Clinic Institutional Animal Care and Use Committee. We used both rats and mice in this investigation in order to determine the consistency of our findings in different species with allogeneic and xenogeneic transplantations of MSCs. The methods were carried out in accordance with the approved guideline.

Animals. Adult male Sprague–Dawley rats weighing 200–250 g, 8–10 weeks old (Harlan, Indianapolis, IN, USA) were used in experiments on opioid tolerance and opioid-induced hyperalgesia. The animals were group housed (2/cage) on a 12 hour light/dark cycle with food and water available ad libitum. The animals were allowed to habituate to the housing facilities for at least 1 week before starting behavioral experiments.

C57BL/6J mice (8–10 weeks, The Jackson Laboratory, Bar Harbor, Maine. USA) were used and were group housed (4–5/cage) in standard cages in a colony room maintained on a 12-hour reversed light/dark cycle. All mice had continuous access to food and water throughout the study. Similarly, mice were handled and adapted to the testing environment for 1 week prior to initiation of the experiments. All behavioral testing procedures were conducted between 08:00 and 13:00 h. Animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee at Cleveland Clinic.

Isolation and culture of MSCs from the bone marrow of rats. MSCs were isolated from the bone marrow as described[55] with minor modifications. Rats (n = 6) sacrificed by CO2 asphyxiation according to Institutional Animal Care and Use Committee (IACUC) guidelines. The femurs and tibiae were removed from six-week-old male Sprague-Dawley rats, and washed three times with sterilized 1xPBS. The ends of the tibia and femur were cut by sharp Scissors. A 25-gauge needle was inserted into the bone marrow to flush out the tissue with a-MEM and filtered through a 100-μm filter mesh (BD Bioscience). The bone marrow (BM) cells were cultured in a-MEM with 16% fetal bovine serum (FBS), 1% L-glutamine, and antibiotic solution (100 u/ml penicillin-streptomycin) in culture flask and incubated at 37 °C with 5% CO2. The medium was replaced after 24 h and every 3–4 days thereafter. MSCs were passaged when they reached 90% confluency by 1 min treatment with 0.05% trypsin and 0.02% EDTA at 37 °C. MSCs were characterized by surface markers through flow cytometry and by differentiation into adipogenic and osteogenic cells. Cell differentiation was tested at passage 4 according to manufacturer's protocol (Rat MSC differentiation kit, sc020, Fisher). MSCs used in all experiments were controlled within passage 5.

Characterization of MSCs by flow cytometry. MSCs were expanded to passage four and were examined for surface marker expression using flow cytometry as previously described[56,57] with modifications. Briefly, cultured MSCs were harvested, washed, and re-suspended in FACS buffer (1% FCS and 0.1% sodium azide in 1xHBSS). After blocking with CD16/CD32 Abs at 4 °C for 30 min, cells were stained for surface markers with directly conjugated Abs in FACS buffer at 4 °C for 30 min. Cells were washed twice and re-suspended in 200–400 μl of PBS for flow cytometry analysis as described before[57]. Abs used in our experiments were CD31PE-CY7 (Clone: 390, eBoscience), CD44HFITC (Clone: OX-49, BD), CD90.1 BV711 (Clone OX-7, BD), CD45APC-CY7 (Clone: OX-1, BD), CD11bPE (Clone: OX-42, BD), CD29BV450 (Clone: HA2/5BD). Flow cytometry analysis was performed with a LSRFortessa cytometer (BD Biosciences) and equipped with CellQuest software (BD Biosciences), and 50,000 events were acquired. Data were analyzed with FlowJo software (Tree Star).

Characterization of MSCs by differentiation. *Adipogenic differentiation.* MSCs were incubated in the completed culture medium supplemented with Rat MSC differentiation kit (sc020, Fisher) for 2 weeks. The medium was changed twice per week. After 2 weeks of adipogenic differentiation, the cells were washed with PBS and fixed in 4% PFA (paraformaldehyde, Sigma) for 1 h at 4 °C, stained for 10–15 minutes at room temperature with a working solution for Oil Red O (Sigma) stain, and rinsed three times with distilled water.

Osteogenic differentiation. MSCs were cultured in the completed culture medium supplemented with Rat MSC differentiation kit (sc020, Fisher). After three weeks of osteogenic differentiation, cells were fixed in 4% PFA (paraformaldehyde, Sigma) for 1 h at 4 °C and stained with 40 mM Alizarin Red (pH 4.1, Sigma) for 10 minutes to visualize calcium deposition.

Induction of OT and OIH. *Acute opioid tolerance.* Rats were injected with equal volume of normal saline (control) or 7.5 mg/kg morphine (experimental group) subcutaneously for 3 days. On the fourth day, rats of both groups received cumulative doses of morphine (0.0, 3.2, 5.6, 8.0, 10.0, and 18.0 mg/kg) as described[58]. Thermal plantar test was used to construct the agonist dose-response curves.

Chronic OT and OIH. Morphine was diluted in sterile saline solution (0.9% NaCl) to a concentration of 7.5 mg/ml. Control group received an equal volume of sterile saline. Morphine sulfate (7.5 mg/kg) was injected subcutaneously daily to induce opioid tolerance. Either saline or morphine was injected at 10:00–11:00 AM.

To evaluate OT, paw withdrawal thresholds (PWTs) (in grams) to mechanical stimulation were measured before and 50 min after each daily morphine injection. The differences between the two measurements reflect the level of tolerance to morphine. A large difference indicates no or low tolerance while a small difference indicates high tolerance. Similarly, thermal stimulation (Hargreave's test) and tail flick test were further used to evaluate opioid tolerance. The response latencies (in seconds) were measured before the initiation of daily morphine injections to establish baseline control values and then measured in various time points after the initiation of daily morphine injections (Figs 2, 3 and 4). The measurements were done in the morning, 30 min after each morphine injection.

OIH was assessed by measuring PWTs to mechanical stimulation. Baseline values were first established 3 days before the initiation of daily morphine injections. PWT values were then measured every other day after the initiation of morphine injections. All measurements were taken in the morning before each morphine injection.

OT and OIH in mice. To induce OT, mice were injected with 10 mg/kg of morphine for 14 days as previously reported with modification[59]. Tail flick tests were performed before the initiation of daily morphine injections to establish baseline values. The response latencies (in seconds) were further measured after initiation of daily morphine injections. The measurements were taken in the morning before and 30 min after each morphine injection. The differences between the two measurements reflect the level of tolerance to morphine. A large difference indicates no or low tolerance while a small difference indicates high tolerance.

OIH was assessed by measuring tail flick test. Baseline values were first established 3 days before the initiation of daily morphine injections. Tail flick latencies were then measured at defined intervals after the initiation of morphine injections.

Intrathecal and intravenous transplantation of MSCs. Intrathecal transplantation of MSCs was performed in the lumbar region, as described by Lu *et al.*[60] with modification. The rat was shaved in lumbar region of the back under anesthesia (40 mg/kg Phenobarbital Sodium), and placed on a rolled pad so the back was arched. After skin disinfection, the L4-L5 lumbar interspace was identified by palpating spinous processes. A needle (27G) was slowly advanced through the skin over the L4-L5 interspace until it reaches the subarachnoid space. Upon confirmation of needle placement in the subarachnoid space by a tail flick, a single dose of cells in 10 ul PBS was injected over a period of one minute.

Intravenous transplantation of MSCs was performed through the tail vein in rats and a lateral retro-orbital approach in mice[61]. One dose of MSCs in 100 ul PBS was injected in each animal.

MSC-TP was performed before the initiation of daily morphine injections to evaluate the preventive effect or after the initiation of daily morphine injections to evaluate the therapeutic effect. For preventive effect, transplantation was performed one day or 7 days before the initiation of daily morphine injections. At each experimental paradigm, rats were divided into three groups, each receiving PBS (Control group), 0.5 million MSCs intrathecally (IT MSC group), or 0.5 million MSCs intravenously (IV MSC group). Each group had 13–14 animals. Behavioral tests were performed on the days as indicated (Fig. 3a).

For therapeutic effect, transplantation was performed 14 days after the initiation of daily morphine injections when opioid tolerance and opioid-induced hyperalgesia have been established. Similarly, rats were divided into three groups, each receiving PBS (Control group), 0.5 million MSCs intrathecally (IT MSC group), or 0.5 million MSCs intravenously (IV MSC group). Each group had 13–14 animals. Mechanical test and tail flick for thermal test were performed on the days as indicated (Fig. 4a).

Behavioral tests. The sensitivity to noxious stimulation was determined by measuring the paw withdrawal thresholds (PWTs) in response to mechanical and thermal stimulation to the right hind paw or by measuring the tail flick latencies in response to a set temperature thermal stimulation to the tail. Animals were handled and habituated before behavioral testing to familiarize them with the environment and to minimize stress. All behavioral tests were performed in the Behavior Core Facility by experienced experimenters who were blinded to the treatment. The behavioral measurements began prior to morphine treatment to determine the baseline values and continued as indicated (Figs 3a and 4a).

Mechanical Sensitivity Testing. Animals were placed in individual 10 × 10 × 15 cm plastic boxes on an elevated metal mesh floor and allowed to acclimate for at least 30 min before test. Mechanical sensitivity was tested using von Frey sensory evaluator filaments (*Stoelting, Wood Dale, IL, USA*)[62,63]. Filaments were applied to the plantar surface of the right hind paw in ascending order of force (0.4–60 grams) until the filament bent and was held there for ~3 seconds or until a paw withdrawal response took place. Upon a paw withdrawal response, the

filaments were applied in descending order, beginning with the next thinner filament until there was no withdrawal response. The threshold was the thinnest filament to evoke a paw withdrawal response. The procedure was repeated three times at 5 min intervals to avoid sensitization and the withdrawal thresholds were averaged and recorded (mean ± SEM).

Thermal Sensitivity testing (*Tail Flick Test*). Sensitivity to thermal stimulation was evaluated by the hot water tail-flick test[64] with modification. The temperature of a digital water bath was set up (52 °C) and confirmed with a glass thermometer. Animals were allowed to acclimate to the laboratory environment for 1 h before testing. After subcutaneously injection of morphine or saline, the animals were returned to their cage. For tail flick test, the animals were restrained with a restrainer (*Harvard Bioscience, Inc., Whitehall, PA*). Approximately 5 min before the test, animals were removed from their cage and allowed to crawl into the restrainer. The tail was marked with ink at one third from the distal tip and submerged in the hot water to the marked level. The cut-off time was limited to 10 s to avoid the tissue damage as measured on a digital laboratory timer. The flick of the tail was recorded as the tail-flick latency in seconds. The test was performed 30 min after morphine injection. Data were calculated as percent maximal possible effect (100% MPE), which was calculated by the following formula: 100% × [(treatment response time – basal response time)/(10 s –basal response time)] = 100% MPE. The group data were expressed as mean MPE ± SEM. The entire behavioral testing was blinded with respect to the treatment groups.

Thermal plantar testing (*Hargreaves test*). Rats were allowed to habituate in the environment for at least 60 min prior to the behavioral test[65]. Each rat was placed in a box (22 × 12 × 12 cm) containing a smooth glass floor (*Stoelting, Wood Dale, IL, USA*)[62,66]. The temperature of the glass was measured and maintained at 27 °C ± 0.5 °C. A heat source (*Stoelting, Wood Dale, IL, USA*) was focused on a portion of the hindpaw and a radiant thermal stimulus was delivered. The stimulus shut off automatically when the hindpaw moved or 20 seconds had passed to prevent tissue injury. The intensity of the radiant heat stimuli was adjusted to obtain either short or long baseline latencies. This allowed quantization of the treatment effect (lengthening of the latency, relative to the baseline values and control groups). In this study, latencies for Hargreaves stimuli at baseline ranged from 7 to 11 s. The experimenters were blinded to group assignments.

The safety measures. The safety measures included food and fluid intake, locomotion, body weight (g), and liver and renal function tests. Since the locomotor function and food and fluid intake were not noticeably different between different treatment groups in our preliminary experiments, we focused on measuring and recording body weight gain (expressed as mean ± SEM) over time among different experimental groups. In addition, we also measured the levels of blood glucose and alanine aminotransferase (*ALT*) to monitor the liver function and blood urea nitrogen (BUN) and creatinine to monitor the kidney function.

Immunohistochemistry and quantitation of immunoreactivity staining. Histology and immunohistochemistry of microglia and astrocytes in the spinal cord were performed as we have described previously[61,67]. Briefly, animals were sacrificed by intracardiac perfusion with ice-cold PBS, followed by 4% PFA solution under deep anesthesia (Phenobarbital Sodium). The lumbar segment of the spinal cord was rapidly dissected and post-fixed in 4% PFA. It was washed with PBS and placed in cryoprotection buffer overnight at 4 °C until it sank. Afterwards, 30-um-thick coronal sections of the spinal cord were cut on a sliding microtome or cryostat machine (Leica Microsystems) and kept in cryostorage buffer at −20 °C.

For immunohistochemistry staining, the tissue slices were rinsed in PBST (1xPBS + 0.05% TritonX-100), blocked by incubation with 5% goat serum and 1% BSA at room temperature for 1 h, and then incubated overnight at 4 °C with primary Abs for microglia specific marker IBA-1 (rabbit anti-mouse/rat IBA-1, 1:5000, WAKO) and astrocyte specific marker GFAP (mouse-GFAP-Cy3, 1:5000, C9205, Sigma). The next day, tissues were incubated with Alexa 594-anti-mouse antibody and Alexa 488-anti-rabbit antibody (Invitrogen) for 1 hour. The immunofluorescence stained tissues were mounted with VECTASHIELD HardSet Anti-fade mounting medium with DAPI (H-1500, Vector lab). Omission of the primary antibody served as negative control. The stained sections were examined with a fluorescence microscope (Leica, Wetzlar, Germany), and images were captured with confocal microscopy (Leica, Germany).

Quantitation of immunoreactivity and cell counting. The quantification of percentage area occupied by immunoreactivity, the number of cells in predetermined area of the superficial dorsal horn (number per unit area), or percentage of immunoreactive cells among total cells were performed as previously described[61,67]. Briefly, 40x pictures of the superficial area of the lumbar spinal cord dorsal horn (L4-6) were captured by confocal microscopy and used for quantification. Digitized images were analyzed with National Institutes of Health ImageJ1.34s software. A thresholding procedure was established to determine the proportion of immunoreactive area within each fixed field of view. These parameters were then held constant for each set of images obtained at equal objectives and light intensities. The data represent the mean immunoreactivity area in the spinal cord horn. The percentage of total number of microglial cells was counted in defined area of the spinal cord dorsal horn (Fig. 11).

MSCs labeling and tracing *in vivo*. MSCs were labeled with Dil (Invitrogen, D-3911) according to manufacturer's instructions. In brief, 2 to 3 million of MSCs were collected and incubated with Dil dye (2 mg/ml) at 1XPBS w/o Ca^{2+} and $Mg2^{+}$ buffer (Invitrogen) at 37 °C water bath for 5 minutes with 2–3 times of shaking. Cells were washed with the 1xPBS and re-suspended in 1xPBS for transplantation. Rats lumbar spinal cord segments, L1–L6 DRGs and sciatic nerve were perfused with 4% PFA indicated below after MSC-TP 1, 5 and 8 days. Low magnification pictures (10x) for the whole spinal cord were captured by anatomy fluorescence microscope with digital camera (Leica, Image Core, CCF, LRI). The tissues were cut into 20 uM section to exam Dil histologically.

Statistical analysis. The paw withdrawal thresholds to mechanical stimulation were expressed as mean ± SEM. The withdrawal thresholds to thermal stimulation (Hargreaves test or tail flick test) were used to calculate % MPE as described and expressed as the mean % MPE ± SEM. The use of % MPE took into account the inevitable differences in baseline values between different groups of animals so that such variability would not affect comparisons between different groups. Body weight and blood tests for liver and kidney functions were expressed as mean ± SEM. Immunoreactivity was expressed as mean ± SEM.

Statistical analyses were made using one way or two way analysis of variance (ANOVA) followed by paired comparisons with Bonferroni corrections when comparisons were made between more than 3 groups. One way analysis of variance (ANOVA) followed by Turkey's multiple comparison test for immunohistochemistry analysis. Graphpad Prism was used for all the analysis. $P < 0.05$ was considered statistically significant ($^{*}P < 0.05$; $^{\#}P < 0.01$; $^{¥}P < 0.001$).

References

1. Relieving Pain in America. (The National Academies Press, 2011).
2. Keating, A. How do mesenchymal stromal cells suppress T cells? *Cell Stem Cell* **2,** 106–108 (2008).
3. Dart, R. C. *et al.* Trends in opioid analgesic abuse and mortality in the United States. *N Engl J Med.* **372,** 241–248 (2015).
4. Mao, J., Sung, B., Ji, R. R. & Lim, G. Neuronal apoptosis associated with morphine tolerance: evidence for an opioid-induced neurotoxic mechanism. *The Journal of neuroscience: the official journal of the Society for Neuroscience* **22,** 7650–7661 (2002).
5. Bekhit, M. H. Opioid-induced hyperalgesia and tolerance. *Am J Ther.* **17,** 498–510 (2010).
6. Vanderah, T. W. *et al.* Tonic descending facilitation from the rostral ventromedial medulla mediates opioid-induced abnormal pain and antinociceptive tolerance. *The Journal of neuroscience: the official journal of the Society for Neuroscience* **21,** 279–286 (2001).
7. Ballantyne, J. C. & Mao, J. Opioid therapy for chronic pain. *N Engl J Med.* **349,** 1943–1953 (2003).
8. Chu, L., Clark, D. & Angst, M. Opioid Tolerance and Hyperalgesia in Chronic Pain Patients After One Month of Oral Morphine Therapy: A Preliminary Prospective Study. *The Journal of Pain* **7,** 43–48 (2006).
9. Chu, L. F. *et al.* Analgesic tolerance without demonstrable opioid-induced hyperalgesia: A double-blinded, randomized, placebo-controlled trial of sustained-release morphine for treatment of chronic nonradicular low-back pain. *Pain* **153,** 1583–1592 (2012).
10. Ellis, A. & Bennett, D. L. Neuroinflammation and the generation of neuropathic pain. *Br J Anaesth* **111,** 26–37 (2013).
11. Schafer, S. *et al.* Influence of intrathecal delivery of bone marrow-derived mesenchymal stem cells on spinal inflammation and pain hypersensitivity in a rat model of peripheral nerve injury. *J Neuroinflammation* **11,** 157 (2014).
12. Wang, X. *et al.* Morphine activates neuroinflammation in a manner parallel to endotoxin. *Proc Natl Acad Sci USA* **109,** 6325–6330 (2012).
13. DeLeo, J. A., Tanga, F. Y. & Tawfik, V. L. Neuroimmune activation and neuroinflammation in chronic pain and opioid tolerance/hyperalgesia. *Neuroscientist* **10,** 40–52 (2004).
14. Ferrini, F. *et al.* Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl(-) homeostasis. *Nat Neurosci.* **16,** 183–192 (2013).
15. Zhang, R. *et al.* Anti-inflammatory and immunomodulatory mechanisms of mesenchymal stem cell transplantation in experimental traumatic brain injury. *J Neuroinflammation* **10,** 106 (2013).
16. Hoogduijn, M. J. *et al.* Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. *Stem cells and development* **16,** 597–604 (2007).
17. Sondergaard, C. S. *et al.* Human thymus mesenchymal stromal cells augment force production in self-organized cardiac tissue. *Ann Thorac Surg.* **90,** 796–803, discussion 803–794 (2010).
18. Hosseini, M., Yousefifard, M., Aziznejad, H. & Nasirinezhad, F. The Effect of Bone Marrow-Derived Mesenchymal Stem Cell Transplantation on Allodynia and Hyperalgesia in Neuropathic Animals: A Systematic Review with Meta-Analysis. *Biol Blood Marrow Transplant* **21,** 1537–1544 (2015).
19. Shibata, T. *et al.* Transplantation of bone marrow-derived mesenchymal stem cells improves diabetic polyneuropathy in rats. *Diabetes* **57,** 3099–3107 (2008).
20. Chen, G., Park, C. K., Xie, R. G. & Ji, R. R. Intrathecal bone marrow stromal cells inhibit neuropathic pain via TGF-beta secretion. *J Clin Invest* **125,** 3226–3240 (2015).
21. Caplan, A. I. & Correa, D. The MSC: an injury drugstore. *Cell Stem Cell* **9,** 11–15 (2011).
22. English, K. Mechanisms of mesenchymal stromal cell immunomodulation. *Immunol Cell Biol.* **91,** 19–26 (2013).
23. Caplan, A. I. & Dennis, J. E. Mesenchymal stem cells as trophic mediators. *J Cell Biochem.* **98,** 1076–1084 (2006).
24. Mayer, D. J., Mao, J., Holt, J. & Price, D. D. Cellular mechanisms of neuropathic pain, morphine tolerance, and their interactions. *Proc Natl Acad Sci USA* **96,** 7731–7736 (1999).
25. Barbash, I. M. *et al.* Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. *Circulation* **108,** 863–868 (2003).
26. Fischer, U. M. *et al.* Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem cells and development* **18,** 683–692 (2009).
27. Harting, M. T. *et al.* Intravenous mesenchymal stem cell therapy for traumatic brain injury. *J Neurosurg* **110,** 1189–1197 (2009).
28. Li, Y. & Lin, F. Mesenchymal stem cells are injured by complement after their contact with serum. *Blood* **120,** 3436–3443 (2012).
29. Matsushita, Y., Omotuyi, I. O., Mukae, T. & Ueda, H. Microglia activation precedes the anti-opioid BDNF and NMDA receptor mechanisms underlying morphine analgesic tolerance. *Curr Pharm Des.* **19,** 7355–7361 (2013).
30. Beitner-Johnson, D., Guitart, X. & Nestler, E. J. Glial fibrillary acidic protein and the mesolimbic dopamine system: regulation by chronic morphine and Lewis-Fischer strain differences in the rat ventral tegmental area. *J Neurochem.* **61,** 1766–1773 (1993).
31. Song, P. & Zhao, Z. Q. The involvement of glial cells in the development of morphine tolerance. *Neurosci Res.* **39,** 281–286 (2001).
32. Raghavendra, V., Rutkowski, M. D. & DeLeo, J. A. The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **22,** 9980–9989 (2002).
33. Sanna, M. D., Ghelardini, C. & Galeotti, N. Activation of JNK pathway in spinal astrocytes contributes to acute ultra-low-dose morphine thermal hyperalgesia. *Pain* **156,** 1265–1275 (2015).
34. Yagi, H. *et al.* Mesenchymal Stem Cells: Mechanisms of Immunomodulation and Homing. *Cell Transplantation* **19,** 667–679 (2010).
35. Maestroni, G. J. *et al.* Neural and endogenous catecholamines in the bone marrow. Circadian association of norepinephrine with hematopoiesis? *Exp Hematol.* **26,** 1172–1177 (1998).
36. Atoui, R. & Chiu, R. C. J. Mesenchymal stromal cells as universal donor cells. *Expert Opinion on Biological Therapy* **12,** 1293–1297 (2012).
37. Patel, A. & Genovese. Potential clinical applications of adult human mesenchymal stem cell (Prochymal®) therapy. *Stem Cells and Cloning: Advances and Applications*, **61** (2011).
38. Inoue, M. *et al.* Comparative analysis of remyelinating potential of focal and intravenous administration of autologous bone marrow cells into the rat demyelinated spinal cord. *Glia* **44,** 111–118 (2003).

39. Pal, R. *et al. Ex vivo*-expanded autologous bone marrow-derived mesenchymal stromal cells in human spinal cord injury/paraplegia: a pilot clinical study. *Cytotherapy* **11,** 897–911 (2009).
40. Hare, J. M. *et al.* A Randomized, Double-Blind, Placebo-Controlled, Dose-Escalation Study of Intravenous Adult Human Mesenchymal Stem Cells (Prochymal) After Acute Myocardial Infarction. *Journal of the American College of Cardiology* **54,** 2277–2286 (2009).
41. Balsam, L. B. *et al.* Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* **428,** 668–673 (2004).
42. Venkataramana, N. K. *et al.* Open-labeled study of unilateral autologous bone-marrow-derived mesenchymal stem cell transplantation in Parkinson's disease. *Translational Research* **155,** 62–70 (2010).
43. Volarevic, V., Arsenijevic, N., Lukic, M. L. & Stojkovic, M. Concise Review: Mesenchymal stem cell treatment of the complications of diabetes mellitus. *Stem Cells* **29,** 5–10 (2011).
44. Kim, E.-J., Kim, N. & Cho, S.-G. The potential use of mesenchymal stem cells in hematopoietic stem cell transplantation. *Experimental & Molecular Medicine* **45,** e2 (2013).
45. Liechty, K. W. *et al.* Engraftment and differentiation of human mesenchymal stem cells after in utero transplantation in fetal sheep. *Journal of the American College of Surgeons* **191,** S96 (2000).
46. Beggs, K. J. *et al.* Immunologic Consequences of Multiple, High-Dose Administration of Allogeneic Mesenchymal Stem Cells to Baboons. *ct.* **15,** 711–721 (2006).
47. P. De Miguel, M. *et al.* Immunosuppressive Properties of Mesenchymal Stem Cells: Advances and Applications. *CMM* **12,** 574–591 (2012).
48. Fehlings, M. G., Vawda, R. & Wilcox, J. Current stem cell treatments for spinal cord injury. *Indian Journal of Orthopaedics* **46,** 10 (2012).
49. Katsuno, T. *et al.* Mesenchymal stem cells administered in the early phase of tumorigenesis inhibit colorectal tumor development in rats. *J. Clin. Biochem. Nutr.* **53,** 170–175 (2013).
50. Mazzini, L. *et al.* Autologous mesenchymal stem cells: clinical applications in amyotrophic lateral sclerosis. *Neurological Research* **28,** 523–526 (2006).
51. Bernardo, M. E. *et al.* Human Bone Marrow Derived Mesenchymal Stem Cells Do Not Undergo Transformation after Long-term *In vitro* Culture and Do Not Exhibit Telomere Maintenance Mechanisms. *Cancer Research* **67,** 9142–9149 (2007).
52. Togel, F. E. & Westenfelder, C. Mesenchymal stem cells: a new therapeutic tool for AKI. *Nat Rev Nephrol.* **6,** 179–183 (2010).
53. Monsel, A. *et al.* Cell-based therapy for acute organ injury: preclinical evidence and ongoing clinical trials using mesenchymal stem cells. *Anesthesiology* **121,** 1099–1121 (2014).
54. Guo, W. *et al.* Bone marrow stromal cells produce long-term pain relief in rat models of persistent pain. *Stem Cells* **29,** 1294–1303 (2011).
55. Lennon, D. P. & Caplan, A. I. Isolation of rat marrow-derived mesenchymal stem cells. *Exp Hematol* **34,** 1606–1607 (2006).
56. Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284,** 143–147 (1999).
57. Liu, L. *et al.* CXCR2-positive neutrophils are essential for cuprizone-induced demyelination: relevance to multiple sclerosis. *Nat Neurosci.* **13,** 319–326 (2010).
58. Eidson, L. N. & Murphy, A. Z. Persistent peripheral inflammation attenuates morphine-induced periaqueductal gray glial cell activation and analgesic tolerance in the male rat. *J Pain* **14,** 393–404 (2013).
59. Bohn, L. M., Gainetdinov, R. R., Lin, F. T., Lefkowitz, R. J. & Caron, M. G. Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* **408,** 720–723 (2000).
60. Lu, Y., Sweitzer, S. M., Laurito, C. E. & Yeomans, D. C. Differential opioid inhibition of C- and A delta- fiber mediated thermonociception after stimulation of the nucleus raphe magnus. *Anesth Analg* **98,** 414–419, table of contents (2004).
61. Liu, L. *et al.* Myelin repair is accelerated by inactivating CXCR2 on nonhematopoietic cells. *The Journal of neuroscience: the official journal of the Society for Neuroscience* **30,** 9074–9083 (2010).
62. Shen, J., Fox, L. E. & Cheng, J. Differential effects of peripheral versus central coadministration of qx-314 and capsaicin on neuropathic pain in rats. *Anesthesiology* **117,** 365–380 (2012).
63. Huang, K. *et al.* A Panel of CpG Methylation Sites Distinguishes Human Embryonic Stem Cells and Induced Pluripotent Stem Cells. *Stem Cell Reports* **2,** 36–43 (2014).
64. Zhou, Q. *et al.* Optimal interval for hot water immersion tail-flick test in rats. *Acta Neuropsychiatr* **26,** 218–222 (2014).
65. Hargreaves, K., Dubner, R., Brown, F., Flores, C. & Joris, J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32,** 77–88 (1988).
66. Shen, J., Fox, L. E. & Cheng, J. Swim therapy reduces mechanical allodynia and thermal hyperalgesia induced by chronic constriction nerve injury in rats. *Pain Medicine* **14,** 516–525 (2013).
67. Liu, L. *et al.* Cutting edge: the silent chemokine receptor D6 is required for generating T cell responses that mediate experimental autoimmune encephalomyelitis. *J Immunol.* **177,** 17–21 (2006).
68. Wexler, S. A. *et al.* Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. *Br J Haematol.* **121,** 368–374 (2003).

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Author Contributions

J.C. and T.Q. formulated the hypothesis. J.C. and L.P.L. designed and supervised the project and prepared the manuscript. Z.H. and J.S. designed most of the behavioral experiments. J.S., H.N.Y., Y.L., L.P.L. and Z.H. isolated and characterized MSCs. Z.H., L.P.L., J.S., K.C., A.L., J.Y. and L.W. performed the transplantation and behavioral experiments. L.P.L. and Z.H. performed immunohistochemistry and histology experiments. H.W. and J.N. performed image quantification. L.P.L., A.L. and J.C. performed statistical analysis and data interpretation. All authors read and approved the final manuscript.

Additional Information

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